Optimizing the Approach for Maintaining Single Muscle Fibers in Culture

By

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A thesis submitted to the Faculty of Graduate and Post-Graduate Studies of the University of Ottawa in partial fulfillment of the requirements of the Degree of Masters of Science

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ABSTRACT

The skeletal muscle is a dynamic tissue that has the ability to change and modify itself to fit the level of required activity; a phenomenon called muscle plasticity. Most studies of muscle plasticity are carried out in situ, a condition for which it is difficult to study and discern between the intrinsic properties of skeletal muscle, the myokines released by muscle fibers and the neurotrophic factors released by neurons innervating skeletal muscles that play various roles in the mechanisms of muscle plasticity. Another approach is to study the morphological and contractile properties of single adult muscle fibers under culture conditions for which one can fully control the level of activity and exogenous factors affecting muscle plasticity. However, the survival of single muscle fiber in culture is very low as most fibers degenerated or supercontracted within 5-7 days. The first objective of this study was to optimize fiber survival in culture. The application of chronic stimulation and β-adrenergic agonists are two major factors that prevent muscle atrophy and loss of force in denervated muscles in situ. So, objective two was to determine if chronically stimulated single fibers in culture also improve fiber survival and contractile characteristic under culture conditions. The third objective was the same for salbutamol, a β2-adrenergic agonist. In regard to the optimization of fiber survival, the Minimum Essential Medium (MEM) was a better medium than Dulbecco’s Modified Eagle Medium (DMEM), changing 50% of the culture medium every two days also improved fiber survival compared to changing the medium every day. Interestingly, inhibiting the proliferation of satellite cells with AraC largely improved fiber survival when fibers were kept under resting conditions, but not when they were chronically stimulated. Finally, under conditions in which proliferation of satellite cells was inhibited, the use of a collagen/laminin mixture as adhering substrate to
improve fiber adhesion to glass coverslip gave rise to a better fiber survival than Matrigel that contains not only collagen and laminin but several growth factors. The results suggest i) that when satellite cells (or fibroblasts) are allowed to proliferate they appear to contribute to the degeneration of fibers under resting conditions and ii) that the release of myokines by skeletal muscle fibers (or cytokines by other cells) likely play a role in fiber survival. Contrary to the situation in situ, neither the chronic stimulation nor salbutamol improved fiber survival and contractile characteristics of muscle fibers in culture suggesting that some important factors in culture are missing to allow chronic stimulation and salbutamol to reduce muscle atrophy and loss of force.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>NaV</td>
<td>Voltage gated sodium channels</td>
</tr>
<tr>
<td>KV</td>
<td>Potassium channels</td>
</tr>
<tr>
<td>Cav</td>
<td>Ca^{2+} channels</td>
</tr>
<tr>
<td>DHPR</td>
<td>Dihydropyridine receptors</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptors</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor digitorum longus</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TA</td>
<td>Tibialis anterior</td>
</tr>
<tr>
<td>ALD</td>
<td>Anterior latissimus dorsi</td>
</tr>
<tr>
<td>CSA</td>
<td>Cross section area</td>
</tr>
<tr>
<td>CP</td>
<td>Creatinine phosphate</td>
</tr>
<tr>
<td>G-6-P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>MDH</td>
<td>Malate dehydrogenase</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cell</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator receptor gamma coactivator-1α</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
</tr>
<tr>
<td>MEF2</td>
<td>Myocyte enhancer factor-2</td>
</tr>
<tr>
<td>TnI</td>
<td>Troponin I</td>
</tr>
<tr>
<td>sMtCK</td>
<td>Sarcomeric mitochondrial kinase</td>
</tr>
<tr>
<td>PGC1β</td>
<td>Peroxisome proliferator-activated receptor gamma co-activator 1</td>
</tr>
<tr>
<td>PGC1α&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>PGC1α knockout mice</td>
</tr>
<tr>
<td>RIP140</td>
<td>Receptor interacting protein 140</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin like growth factor</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>mTOR</td>
<td>Rapamycin</td>
</tr>
<tr>
<td>GSK3b</td>
<td>Glycogen synthase kinase 3b</td>
</tr>
<tr>
<td>FoxO</td>
<td>Forkhead box</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene related peptide</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine triphosphate</td>
</tr>
<tr>
<td>Gs</td>
<td>G-stimulatory protein</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinas A</td>
</tr>
<tr>
<td>FDB</td>
<td>Flexor muscles belong</td>
</tr>
<tr>
<td>AraC</td>
<td>Cytosine β-D-arabinofuranoside</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
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<td>Abbreviation</td>
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<td>--------------</td>
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</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>Matrigel</td>
<td>Basement Membrane Matrix</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>[Ca^{2+}]_i</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>N- CAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>Pax-7</td>
<td>Piried-bax transcription factor</td>
</tr>
<tr>
<td>mV</td>
<td>Millivolt</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond</td>
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ACKNOWLEDGEMENTS

First and foremost I would like to express my gratitude to my supervisor, Dr. Jean-Marc Renaud. It has been an honor for me to work with him. He has taught me how to expand my vision and my way of thinking in science and in life as well. I appreciate all the efforts he invested in me, the time he dedicated to me, ideas, guidance, and support to make my M.Sc. experience productive and stimulating. The joy and enthusiasm he has for his research was contagious and motivational for me as well as for each member working with him. Also, I would like to extend my thanks and appreciations for Dr. Jocelyn Côté and Dr. William Staines for their insightful advices and continued interests.

I would like to thank my family for all their love and encouragement. Thanks for my parents who raised me with love and supported me throughout my pursuits. Thank you my lovely sisters. As well as lab mates, Tarek, Amanda, Erik, and Wei, thank you for your friendship, thank you for your help. It has been a great pleasure to work with you all. Thanks to everyone who has helped me along the way. At last but not least, I would like to thank my fiancé for his unconditional support, encouragement, and faith, until this moment. I could not have done it without you.

Lastly, I gratefully acknowledge the funding source (King Abduallah scholarships program, Kingdom of Saudi Arabia: represented by the Saudi Cultural Bureau, Canada) that made my M.Sc. possible. I was honored to receive a full scholarship to finish my graduate studies.
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INTRODUCTION

Muscle contraction is a process that starts when motorneurons release acetylcholine in the synaptic cleft of the neuromuscular junction. Acetylcholine binds to ligand nicotinic receptors, which are non-selective cation channels, located on the post synaptic membrane of muscle fibers. This allows for an increased conductance of sodium and potassium, which depolarizes the sarcolemma (muscle cell membrane) because the Na\(^+\) influx is greater than the K\(^+\) efflux. When a potential has reached threshold, an action potential is generated. The action potential is defined as a short event during which the cell membrane rapidly depolarizes from its resting level of -70 mV to +30 mV before it repolarizes back to -70 mV. Voltage gated sodium channels (NaV) and potassium channels (KV) are closed when the membrane potential is -70 mV. When the membrane potential depolarizes to a threshold of about -60 mV, NaV channels become activated. As they open, Na\(^+\) influx causes the action potential depolarization phase. Eventually, NaV channels become inactivated while KV channels open and allow for K\(^+\) efflux, both events being important for the repolarization phase back to -70 mV (McGraw, 1999).

Action potential spread along the sarcolemma and down transverse tubules (T-tubules) where voltage gated Ca\(^{2+}\) channels (Ca\(_{v}\)), also known as L-type Ca\(^{2+}\) channels and dihydropyridine receptors (DHPR). DHPR are physically connected to ryanodine receptors (RyR), which are the Ca\(^{2+}\) release channels in the sarcoplasmic reticulum (SR) where Ca\(^{2+}\) is stored. When action potential excites the DHPR, RyR are physically activated via protein–protein interaction and RyR open to release Ca\(^{2+}\) from the SR. Once Ca\(^{2+}\) is released, it binds to troponin located on the thin filament (actin) of the sarcomeres. The troponin allosterically moves tropomyosin, another protein, on the thin filament. Under normal conditions, tropomyosin sterically obstructs the
binding sites for myosin on actin; once Ca$^{2+}$ binds to the troponin C and causes an allosteric change in the troponin conformation, it allows troponin T to move tropomyosin away from the myosin binding site located on the actin. Then, myosin binds to actin, and pulls the actin filaments toward the center of the sarcomere to generate force or work while using ATP as energy. Eventually, actin detaches from myosin and the cycle is repeated as long as ATP and Ca$^{2+}$ are available. Relaxation occurs when there is no more action potential on the cell membrane allowing the Ca$^{2+}$ ATPase to pump Ca$^{2+}$ back into the SR (McGraw, 1999).

Skeletal muscle fibers are not homogeneous. They express various fiber types, each with specific contractile and biochemical characteristics to meet various tasks that skeletal muscles provide. Based on the myosin isoform expression, skeletal muscle fibers are first divided into slow twitch (type I) and fast twitch (type II) fibers. Type II is further categorized into type IIA, IIB and IIX fibers. In general, type I and IIA fibers have high oxidative capacity and low glycolytic capacity. Accordingly, these fibers contain a large number of mitochondria and blood vessels, and have high myoglobin content. They have a low supply of creatinine phosphate, low glycogen content, and a high stores of triglycerides. They are very efficient at using oxygen to generate ATP from glucose and fatty acids and can be active for long periods of time before they fatigue. Therefore, those fibers are important in maintaining posture and helping athletes run a marathon or bicycle for hours. By contrast, type IIB fibers have the lowest oxidative capacity and highest glycolytic capacity as they contain fewer mitochondria, less myoglobin, fewer blood vessels and a large amount of glycogen. These fibers fatigue much more quickly than type I and IIA fibers, and thus are much better at generating short bursts of strength or speed such as weightlifting and sprint running. Finally type IIX fibers have intermediate properties between IIA and IIB.
Skeletal muscle fiber type composition is not static, but dynamic, which means muscles have the ability to change and modify themselves to fit the level of a given activity. Training in athletes leads to remarkable phenotypic modifications that maximize muscle performance for a given type of activities. This phenomenon is known as muscle plasticity and the type of modifications depend on the exercise pattern. Skeletal muscle plasticity is thus defined as the adaptive response to physiological demands and/or pathological conditions inducing changes in fiber type content within a muscle (Zierath & Hawley, 2004).

Most studies on muscle plasticity are carried out in vivo (BULLER et al., 1960; Romanul, 1967; Chi et al., 1986; Henriksson et al., 1986). This is a condition for which muscle plasticity depends not only on the intrinsic characteristics of muscle but also on exogenous factors such as hormones, neurotrophic factors and myokines. To better understand the intrinsic properties of muscle plasticity one requires an approach in which skeletal muscle fibers are isolated. This can be achieved when muscle fibers are first separated from each other by trituration after a collagenase digestion and incubated over time. Such an approach is important to control which exogenous factors to which muscle fibers are exposed. However, very few studies have so far used single muscle fibers in culture (Bischoff, 1975; Bekoff & Betz, 1977a; Jay & Barald, 1985). So, one objective of this study was to document the best approach to maintain muscle fibers in culture by measuring the morphological and physiological changes that occur when skeletal muscle fibers are incubated over a long periods of time. This was necessary because, in the first experiment carried out for this study, fibers could only be maintained for 5 days. Chronic stimulation and catecholamines are two factors that reduce atrophy in denervated fibers in vivo. Fibers maintained in culture behaved like denervated fibers (Bekoff & Betz, 1977a). So, the next two objectives were to determine whether chronic stimulation (objective 2) and catecholamines
(objective 3) also reduce atrophy and the loss of contractility of fibers maintained in culture. If culturing muscle fibers in vitro is to be a good approach to study muscle plasticity, then these experiments were important to demonstrate that the effects of chronic stimulation and catecholamines in vitro are similar to those in vivo. Considering the small amount of literature discussing adult single fiber in culture, the following Introduction will mainly examine the mechanisms of skeletal muscle plasticity.

MUSCLE PLASTICITY: PHYSIOLOGICAL AND BIOCHEMICAL CHANGES

Muscle plasticity was first observed with cross-innervated skeletal muscle experiments (Buller et al., 1960; Romanul, 1967). The extensor digitorum longus (EDL) muscle is primarily composed of fast twitch type IIB and IIX fibers while slow soleus muscle primarily contains type I and IIA fibers. Furthermore, EDL is innervated by motorneurons firing at frequencies of 90-110 Hz for a mean time periods of 0.83 sec (Hennig & Lømo, 1985). By contrast, soleus is tonically activated by motorneurons; i.e., low frequency (10-30 Hz) with long bursts varying between 300 and 500 sec (Hennig & Lømo, 1985; McGraw, 1999). Following cross-innervation in which soleus is innervated by the EDL motorneuron and vice versa for EDL, the speed of contraction of EDL muscle slowed by 46% (Eken & Gundersen, 1988) whereas that of soleus muscle increased by 38% over a 3 month period (Romanul, 1967). The change in contraction speed is associated with change in fiber type composition. In EDL, there was a reduction in the proportion of glycolytic fibers type IIB by 98% and type IIX by 81% and increases in type I by 85% and IIA by 10% (Mira et al., 1992). For soleus muscle, there was no significant change in fiber type distribution (Gauthier et al., 1983).

Studies of cross-innervation suggest that skeletal muscle fiber type composition and thus physiological properties can be modified either by change in muscle activity level (intrinsic
properties) or by neurotrophic factors because motorneurons not only release acetylcholine to stimulate muscle contraction but also release other factors that affect muscle gene expression. In human and animal models, the types of changes in muscle phenotype have also been shown to be different between endurance and strength training.

**ENDURANCE TRAINING**

Endurance training is the type of exercise for which the goal is to increase fatigue resistance in order to sustain long periods of muscle activity. This is the typical type of training for athletes involved with long-distance running, bicycling, and swimming. The type of exercises varied among studies but involved low-load exercises such as free weight exercise. For long exercise duration, muscles need to generate ATP for a long time. Such an energy demand requires an increased activity of aerobic metabolism. Indeed, this kind of biochemical adaptation has been reported in humans (Pernow & Saltin, 1971) and animals (Holloszy, 1967; Baldwin et al., 1972). For example, 2 months of training on bicycle ergometers (40 min/day for 4 days/week) is sufficient to enhance the activity of oxidative enzymes such as succinate dehydrogenase by 23-40% and cytochrome oxidase by 40% in quadriceps femoris muscle (Jansson & Kaijser, 1977; Andersen & Henriksson, 1977). Experiments involving mice and rats running on treadmills for 2 hours a day for 12-16 months resulted in a twofold increase in muscle mitochondria content (Baldwin et al., 1972; Winder et al., 1974). Associated with increases in mitochondrial content, there were increases in oxidative enzyme activity and ATP production. The capacity to oxidize fat and carbohydrates increases twofold (Holloszy, 1967; Molé et al., 1971; Baldwin et al., 1972) as the activity of tricarboxylic acid cycle (TCA) enzymes including citrate synthase (Holloszy et al., 1970; Winder et al., 1974) and NAD-specific isocitrate dehydrogenase (Holloszy et al., 1970), all increase by twofold. Activity of other enzymes also increases but to a lower extent such as 50-
60% for glutamate dehydrogenase (Holloszy et al., 1970) and malate dehydrogenase (Holloszy et al., 1970; Molé et al., 1973). Furthermore, two fold increase in glycogen concentration was also reported (Henriksen & Halseth, 1994). In order to match the increased demand for oxygen, muscle capillary density increases by 20% (Jansson & Kaijser, 1977; Andersen & Henriksson, 1977).

Endurance training also results in changes of fiber type distribution toward more fatigue resistance type I fibers. For instance, following 6 weeks of running exercise, the number of type I fibers increased by 18%, concomitantly with decreases in types IIA and IIX fibers by 12% and 7%, respectively (Russell et al., 2003). Chronic stimulation of innervated rabbit tibialis anterior (TA) muscles with 0.2 ms pulses at 20 Hz for 12 hours daily, an alternative approach mimicking endurance training in animals, reduces the proportion of type IIB fibers from 25% to 10% and IIA fibers from 28% to 20%, over a 20 day period. Appearances of hybrid fibers were also reported such as type I-IIA fibers (10%) and type IIA-IIB- fibers (60%). After 30 days of chronic stimulation, all type IIB had disappeared and type IIA, I-IIA and I fibers increased to 60%, 25%, and 10%, respectively (Pette, 1998). Based on some studies, it has been suggested that the sequential change in fiber type isoforms with exercise or chronic stimulation at low frequency occurs in the order of IIB→ IIX →IIA →I fibers (Pette, 1998; Windisch et al., 1998).

STRENGTH TRAINING

The second type of adaptation is the ability of muscles to overcome a resistance by exposing muscles to strength training. Strength training is a type of exercise for which the aim is to increase force or power by increasing muscle mass. In humans, weightlifting and sprint running are typical examples of activities which have a need for such training.
The primary consequence of skeletal muscle resistance training is an increased muscle mass; i.e., hypertrophy. In humans, exposing muscles to heavy resistance training (35-38 sessions/week) for 12-14 weeks leads to an increased muscle mass up to 10-13% while cross sectional area (CSA) of type I and II fibers increased by 10-28% and 13-17%, respectively (McCall et al., 1996; Trappe et al., 2000; Aagaard et al., 2001; Holm et al., 2008). The fibers’ CSAs for types I and II fibers continues to increase if the training lasts another 5-6 months by 31 and 39%, respectively (MacDougall et al., 1980). Increased cross-sectional area is related to an increased rate of protein synthesis and decreased rate of protein degradation (McCall et al., 1996). As a result of greater CSA, there are increases in force production by 15-25% after 12-14 weeks of resistance training (McCall et al., 1996; Aagaard et al., 2001).

Among all these studies, very few have looked at changes in fiber type distribution. In two studies (Trappe et al., 2000; Holm et al., 2008), 12 weeks of resistance training increased, in quadriceps and vastus lateralis muscle, the proportion of fiber type I and IIA by 13-27% and 7%, respectively. Concomitantly the proportion of type IIX, I/IIA and IIA/IIX fibers decreased by 57%, 55% and 28%, respectively.

Weightlifting or sprint running requires a very high rate of energy production for short periods of time. Such demands are best met with muscles with high energy phosphate reserves and an increase in glycolytic capacity and not oxidative capacity such as mitochondria and oxidative phosphorylation which takes time to be activated. Resistance training increases concentrations of energy sources such as ATP by 18%, creatinine by 39%, CP by 22%, and glycogen by 66% (MacDougall et al., 1977). It also corroborates with increases in glucose uptake by 10 fold, glucose-6-phosphate (G-6- P) by 4 fold, and lactate, the end product of glycolysis, by 16% (Tesch et al., 1986; Essén-Gustavsson & Tesch, 1990). Moreover, as a result of the
increased fiber size, capillary density per fiber increases for type I and II fiber by 12% and 21%, respectively (McCall et al., 1996).

DECREASED MUSCLE ACTIVITY

A reduction in muscle activity also leads to changes in skeletal muscle. Clinically, a reduction in muscle activity occurs following spinal cord injury, complete denervation or suspension such as wearing a cast. Under those conditions, skeletal muscles are going through a process known as atrophy. Muscle atrophy is a sequence of events including loss of muscle mass, reduction in fiber diameter, disorganization in intracellular structures and decrease in force generation (Tower, 1935). Over time, there are increases in connective tissue followed by progressive fiber necrosis and replacement of muscle mass by fat and fibrous connective tissue.

Atrophy is primarily due to an increased rate of protein degradation and decreased rate of protein synthesis that eventually cause reduction in overall muscle size. As a consequence of the protein loss, a reduced muscle activity up to one month leads to a 7% decrease in muscle mass and up to 40-65% decrease in muscle CSA (Ingalls et al., 1999; Carlson et al., 2002; Ashley et al., 2007b; Adhihetty et al., 2007). 1-2 months after a spinal cord injury in a human, the reductions in CSA for type I, IIA and IIX fibers were 40%, 51% and 49%, respectively (Scelsi, 2001). Another study also reports that denervation of rat TA muscle for 14 days reduces protein import synthesis in subsarcolemmal space by 50% and intermyofibrillar space by 29% (Singh & Hood, 2011). Associated with lower muscle mass, the maximum force decreases by 24-50% after one month of muscle denervation (Ingalls et al., 1999; Carlson et al., 2002).

Some studies show a shift in fiber type distribution from type I fibers toward type II fibers. For example, after 10 months following a spinal cord injury, the proportion of type I and IIA had been reduced by 6-16% and 30-33%, respectively. Concomitantly, the proportion of type
IIX fibers had increased by 35-65% (Scelsi, 2001). Another study reported that after 6 months of denervation type I fibers were completely lost while the number of type II fibers increased (DelGaudio & Sciote, 1997).

Denervation accelerates the rate of protein degradation in mitochondria as well (Phillips et al., 2009) resulting in smaller mitochondria; i.e., a 43% reduction of their normal size (Scelsi, 2001). This leads to a reduced oxygen consumption and induces oxidative stress. Production of reactive oxygen species (ROS) increases 5 to 7.5 fold, and the latter activates pro-apoptotic factors (Zammit et al., 2006; Powers et al., 2010). There are also reductions in the number of capillaries that supply muscle fibers by 20% (Scelsi, 2001). All those changes lead to myonuclear loss and eventually death for some muscle fibers (Borisov & Carlson, 2000; Borisov et al., 2001). Reduction in glycogen level has also been reported. For instance, a 1 day denervation of rat hemi-diaphragms results in a 50% reduction in glycogen synthesis and glucose transport (Smith & Lawrence, 1984). After two weeks of denervation, the ATP consumption rate decreases in all fiber types and the reduction was greater in type IIX fibers (Sieck et al., 2007). Denervation also reduced resting membrane potential from -70/-75 mV to -63/-55 mV (Albuquerque et al., 1971; Vyskočil et al., 1973; Bray et al., 1976).

REVERSING MUSCLE ATROPHY BY CHRONIC ELECTRICAL STIMULATION

Chronic stimulation is an approach used to mimic physical training and is now being used in the field of rehabilitation as an attempt to reverse muscle atrophy in denervated muscles. A 28–33 day period of chronic stimulation of 3 day denervated rat EDL (25 ms train of pulses at 150 Hz trains every 15 s) and soleus (200 ms train of pulses at 20 Hz trains every 30 s) maintained muscle mass, CSA and force production at levels close to those of normal muscles (Eken & Gundersen, 1988). A ten week denervation of rabbit TA muscle resulted in a reduction in muscle
CSA to 39% of its original size (Ashley et al., 2007b). Then, when chronically stimulated for 2 weeks, CSA of TA muscles increased from 39% to 66% while muscle weight is restored to a value not significantly different from the contralateral innervated normal TA. It suggests that chronic stimulation promotes the protein synthesis pathway and can be a treatment against muscle atrophy by inducing hypertrophy. With regards to force generation, 10 weeks of denervation reduced maximum force generation to 27% of normal. Despite full recovery of muscle mass, chronic stimulation increased specific force to only 50% of normal level (Ashley et al., 2007b). So, chronic stimulation seems to be inducing full recovery of muscle weight but did not fully restore force which suggests that increased muscle activity alone is not enough to completely recover some of the physiological properties such as force. It is possible that missing neurotrophic factors may be one of the reasons.

Chronic electrical stimulation also shifts fiber type distribution back toward type I fibers in denervated muscle. For example, simulated rat EDL immediately after denervation for 3 weeks at 20 Hz for 10 s every 20 s increased the number of type IIA fibers by 75% and type I fibers by 15%, whereas type IIB and IIX fibers disappeared (Windisch et al., 1998). It should be noted that the change in fiber type distribution in denervated muscle with chronic stimulation at low frequency is basically similar to the changes observed with endurance training or stimulation of innervated muscle (Pette, 1998; Windisch et al., 1998).

Chronic stimulation enhances the oxidative capacity of denervated fibers as well. For instance, stimulated rabbit TA muscle at 10 Hz every 1 s for 2-5 weeks increased the activity of succinate dehydrogenase (SDH), citrate synthase and malate dehydrogenase (MDH), which are enzymes of the Krebs’s cycle, by 6 to 12 fold. Concomitantly, there are decreases in glycolic enzyme activity, especially lactate dehydrogenase (LDH) and phosphofructokinase by 70-90%
MECHANISM OF MUSCLE PLASTICITY

The mechanisms involved in fiber plasticity are not fully understood. They depend in part on the intrinsic properties of muscle fibers and in part on exogenous factors that are released by motorneurons and sympathetic nerves. More recently, it has become evident that many of the neurotrophic factors are also released by muscle fibers themselves and are called myokines acting in an autocrine fashion.

INTRINSIC PROPERTIES OF SKELETAL MUSCLE

Adult skeletal muscle undergoes conversion between different fiber types in response to changes in muscular activity (Pette, 1998; Olson & Williams, 2000). A major mechanism that activates the type I fiber program involves the calcium-calmodulin, calcineurin and nuclear factor of the activated T cell (NFAT) pathway.

Calcineurin is a Ca\textsuperscript{2+}-calmodulin dependent protein phosphatase, which is activated upon an increase in myoplasmic [Ca\textsuperscript{2+}]\text{[i]} level. [Ca\textsuperscript{2+}]\text{[i]} binds to calmodulin to form a Ca\textsuperscript{2+}-calmodulin complex, which binds to and activates calcineurin. Activated calcineurin then dephosphorylates cytosolic NFAT, a transcription factor. Once dephosphorylated NFAT is translocated into the nucleus and with other transcriptional factors, such as myocyte enhancer factor-2 (MEF2), it promotes type I fiber expression (Naya et al., 2000).

In a lymphocyte cell, NFAT activation by Ca\textsuperscript{2+} is amplitude and duration dependent (Dolmetsch et al., 1997). A brief Ca\textsuperscript{2+} spike lasting 3 min or less only caused a transient activation and nuclear translocation of NFAT. A prolonged, even if very small increase in intracellular calcium concentration ([Ca\textsuperscript{2+}]\text{[i]}), on the other hand, activates NFAT for a longer time period (Dolmetsch et al., 1997). It has therefore been assumed that the extent of NFAT activation is...
greater in slow muscles such as soleus because when they are repetitively stimulated at low frequencies they have chronic and small increases in $[\text{Ca}^{2+}]_i$ (Dolmetsch et al., 1997; McGraw, 1999). Conversely, nFAT is less active in type II fibers for which short ($<200$ msec long) bursts of $[\text{Ca}^{2+}]_i$ normally occurs.

Transgenic mice that overexpress constitutively activated calcineurin have a greater number of type I fibers compared to wild type mice (Naya et al., 2000). Furthermore, the effect of the calcineurin NFAT pathway is not limited to just the regulation of myosin type I isoform expression; NFAT pathway also up-regulates the expression of type IIA isoform and down-regulates the expression of myosin type IIB and IIX isoform. Conversely, inhibition of calcineurin activation by using cyclosporin A promotes slow-to-fast fiber transformation (Chin et al., 1998). Furthermore, transgenic mice that overexpress MCIP1, a calcineurin inhibitory factor, have normal fiber type distribution at birth, but after 7 days slow fibers start to disappear and by day 14 all slow fibers have switched to type IIA fibers (Oh et al., 2005). Thus, the calcineurin NFAT pathway concomitantly drives slow fiber expression and inhibits fast fiber expression.

There are also other intracellular signaling pathways that not only affect the gene expression of contractile protein isoforms but also affect the gene expression of metabolic enzymes. The most studied one is Peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC1α). The primary role of PGC1α is to initiate mitochondrial biogenesis. PGC1α expression increases after exercise training. Following 6 weeks of running exercise, PGC1α expression had increased by 2.7 fold in muscle fibers. PGC1α expression was greater in type IIA fibers than in type I or IIX fibers by 2.8 fold and 1.5 fold, respectively (Russell et al., 2003). PGC1α knockout mice (PGC1α−/−) exhibit declines in mitochondrial number in soleus muscle consistent with defects in mitochondria biogenesis genes, especially those genes that are involved in the electron
transport chain (Leone et al., 2005). Furthermore, fiber type composition shifts from oxidative type I and IIA toward type IIX and IIB fibers in PGC1α−/− mice (Handschin et al., 2007). PGC1α−/− mice also exhibit a reduction in the capacity for endurance training and fatigue resistance from 24% to 14% following treadmill running which causes fiber damage (Leone et al., 2005). In addition, as a response to muscle disuse or denervation, muscles atrophy is associated with a decrease in PGC1α expression. A lack of PGC1α results in increased FoxO expression, a transcription factor involved in loss of muscle mass (Sandri et al., 2006), which will be discussed later.

Much less is known about the intracellular signaling pathway regulating the expression of type IIA, IIB and IIX fibers. There is, however, a co-activation factor known as Peroxisome proliferator-activated receptor gamma co-activator 1β (PGC1β), which appears important for the expression of type IIX fibers. It also activates the MEF family of transcription factors to stimulate type IIX fibers expression. All TA muscle fibers of transgenic mice overexpressing PGC1β in skeletal muscle are of type IIX, whereas, in wild type TA, the proportion of type IIX fibers ranges between 15 and 20% (Arany et al., 2007). Furthermore, PGC1β increases the transcription of mitochondrial genes. Overexpressed PGC1β increases expression of electron transport chain genes such as cytochrome C, nduf5 (a member of the NAD dehydrogenase complex) and atp50 (a member of the cytochrome oxidases complex) by 5 fold in quadriceps muscle of transgenic mice compared to wild type (Arany et al., 2007). Increases in mitochondrial gene expression suggest an increased oxidative capacity in transgenic mice. Therefore, these transgenic animals can run for longer with higher workloads than wild-type animals.

Receptor-interacting protein 140 (RIP140) is one transcriptional repressor co-factor that contributes to the glycolytic regulatory pathways through nuclear receptors. It increases
glycolytic capacity and regulates fat storage in adipose tissue (Leonardsson et al., 2004). On the other hand, RIP140 down regulates genes expression of oxidative phosphorylation genes (Powelka et al., 2006). For example, in transgenic mice that overexpress RIP140, the gene expression of succinate dehydrogenase decreases in soleus muscle whereas the gene expression of AMPK increases. By contrast the expression of succinate dehydrogenase and mitochondrial DNA increases by 2 fold in gastrocnemius and EDL muscle in RIP140 knockout mice (Seth et al., 2007). Increases in oxidative capacity in knockout mice are associated with increases in the proportion of type IIA and IIX fibers and with decreases in type IIB fibers especially in EDL (Seth et al., 2007). That study supported the possibility of involving RIP140 in regulation expression of glycolytic fibers during strength training.

**NEUROTROPHIC FACTORS AND MYOKINES**

Neurotrophic factors are small proteins that were first identified in embryonic neuronal cells. These factors play a major role in the central nervous system (CNS) plasticity such as neuron development, survival, maintenance, differentiation of glia cells and regulation of synaptogenesis and synaptic plasticity in mature neurons (Lillien & Raff, 1990; Chao, 2003). Based on their function in the CNS, neurotrophic factors are divided into 5 families which are: insulin like growth factors (IGF), neurotrophins, glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) and hepatocyte growth factors (HGF) (Sendtner et al., 2000). However, while motorneurons also secrete neurotrophic factors that can modulate gene expression, it has become evident that skeletal muscle also releases the same factors, which in this case are called myokines and are often a different isoform from those released by neurons (Sakuma & Yamaguchi, 2011). Here, a few of these neurotrophic factors and myokines will be discussed.
**Insulin like growth factor (IGF-1)** is synthesized in several tissues including those of the pituitary gland, liver, skeletal muscle and nervous system (Recio-Pinto *et al.*, 1986; Yang *et al.*, 1995). In peripheral nerves, IGF-1 is mainly released by Schwann cells, which are cells that are wrapped around axons in the peripheral nervous system (Cheng *et al.*, 1996), and act on motorneurons to promote neuronal survival (Neff *et al.*, 1993; Hughes *et al.*, 1993), the myelination process (Jessen *et al.*, 1994) and nerve regeneration. IGF-1 plays a role in the regulation of skeletal muscle growth through IGF-1 receptors. The IGF-1 signaling pathway increases protein synthase and decreases protein degradation through AKT kinase, also called protein kinase B (PKB). The key to the AKT regulatory pathway is phosphatidylinositol-3 kinase (PI3K). IGF-1 stimulates PI3K to phosphorylate AKT (active form of AKT). Phosphorylation of AKT is required to initiate several protein synthesis pathways such as the activation of the kinase in the mammalian target of rapamycin (mTOR) signalling pathway and the inactivation of the glycogen synthase kinase 3b (GSK3b) signalling pathway (Schiaffino & Mammucari, 2011a). In 2 week old transgenic mice that overexpress active AKT, the CSA of gastrocnemius muscle increased by 2-fold relative to wild type mice (Izumiya *et al.*, 2008). Furthermore, exposing gastrocnemius muscle to exogenous activated AKT, increases gastrocnemius CSA by 50% over 3 weeks and specific force by 15% (Blaauw *et al.*, 2010). Furthermore, IGF-1 has been shown to induce hypertrophy by stimulating the proliferation of satellite cells, cells that are located between basal lamina and sarcolemma. Satellite cells are activated to proliferate in order to replace fibers that have been damaged or to add nuclei in skeletal muscle fibers during hypertrophy (Vandenburgh *et al.*, 1991).

IGF-1 also prevents protein degradation by inhibiting the Fox family of transcription factors for atrophic genes (Manning & Cantley, 2007; Schiaffino & Mammucari, 2011b). Fox factors are
necessary for transcriptional regulation of atrophy factors such as the atrophy F-box (MAFbx) and muscle ring finger 1 (MuRF1). They are also important for transcriptional regeneration of microtubule associated protein 1 light chain 3 (LC3), which, together with BCL2/adenovirus E1B interacting protein 3 (BNIP3) are required for activation of the autophagy lysosome pathway by increasing levels of lysosomal protease Cathepsin L (Henderson et al., 1994; Schiaffino & Mammucari, 2011a). In 5 week old transgenic mice that overexpress Foxo-1, body mass was 10% less than wild type. Also Northern blot analysis showed an increase in Cathepsin L expression in quadriceps muscle compared to control (Henderson et al., 1994). IGF-1 inactivates this pathway by phosphorylating the Fox transcription factors. Another way to stimulate protein degradation by Fox factors is by inhibiting mTOR activation.

**Brain-derived neurotrophic factor** (BDNF) belongs to the family of neurotrophins. In the nervous system, BDNF is expressed by Schwann cells. BDNF binds to P75NTR or trk-B receptors in motorneurons. The main functions of BDNF are to maintain and develop motorneuron survival after nerve lesion and to maintain postsynaptic receptors at the neuromuscular junction (NMJ) (Gonzalez et al., 1999). BDNF is also expressed by skeletal muscle and also acts on motorneurons to maintain their survival and to provide regeneration after damage. A Study has shown that chronic stimulation of denervated muscles produces BDNF to stimulate nerve terminal outgrowth and differentiation (Funakoshi et al., 1995).

BDNF also acts on muscle fibers themselves in an autocrine fashion. It functions as a hypertrophic factor and stimulates protein synthesis by activating the AKT/mTOR signaling pathway via tropomyosin related kinase B (TRK-B) receptors (Sakuma & Yamaguchi, 2011). It is also involved in myogenesis and fiber regeneration. After muscle injury, BDNF expression increases at the same time as satellite cells are activated. In BDNF knockout mice (BDNF−/−), the
expression of Pax7, a satellite cell marker, was significantly decreased by 30% in soleus muscle. It further resulted in abnormal proliferation and differentiation of myotubes as well as a reduced fiber size by 38% compared to wild type (Clow & Jasmin, 2010). By contrast, treatment of BDNF/ mice with exogenous BDNF (20 ng/ml) produces normal fibers with normal fiber size. BDNF deficient mice also exhibit delay in fiber regeneration following injury as well as delay in expression of regeneration markers such as myogenin, embMXHC and MyoD in soleus and EDL muscles. After muscle injury, these markers are expressed at a low levels at day 5, whereas, in wild type mice, markers reached a maximum level by day 2 (Clow & Jasmin, 2010). However, muscle activity also regulates the level of BDNF expression. Brief treadmill running bouts over 5 days increase DBNF mRNA expression by 5-fold in rat soleus muscle (Gómez-Pinilla et al., 2001; Cuppini et al., 2007), while denervation for 2 weeks causes a decrease in BDNF expression in soleus and gastrocnemius by 75% and 90%, respectively (Wehrwein et al., 2002).

Together, these studies suggest that BDNF plays a major role in satellite cell activation and that its expression is activity dependent.

**Glial cell line-derived neurotrophic factor (GDNF)** is another neurotropic factor. It was isolated from a rat glioma cell-line as a trophic factor for dopaminergic neurons in embryonic midbrain cells. Later on, it was found that GDNF is also expressed by Schwann cells and skeletal muscle. GDNF binds to a GDNFα receptor to promote motorneuron branching, to enhance spontaneous neurotransmitter release and the Ach receptor insertion at the NMJ (Yang & Nelson, 2004). A study reported that treating neurons with 1 ng/ml of GDNF for 3 days in culture induced an increase in the total length of motor axon by 83% and Ach released by 54% at NMJ (Wang et al., 2002). That suggests that GDNF plays a major role in NMJ development. Expression of GDNF in skeletal muscle changes in response to the level of muscle activity. The
amount of GDNF expressed during low-intensity exercise (10 m/min, 45 min/day, 2 weeks) increased in the soleus muscles from 3.1 to 4.4 (pg/mg tissue), while the average endplate area increased by 20%. However, in EDL, the concentration of GDNF decreased from 2.3 to 1 (pg/mg tissue) corresponding to a 14% decrease in the average endplate area (McCullough et al., 2011). Thus, expression of GDNF is activity and fiber type dependent. All the above studies provide strong evidence for the importance of neurotrophic factors and/or myokines in maintaining normal muscle properties including with denervated muscles.

**Nerve growth factor (NGF)** is expressed by motorneurons and skeletal muscles as well. It plays a role in the development of the nervous system (Bradshaw et al., 1994). In skeletal muscle, NGF stimulates protein synthesis by activating ErbB receptors, present at the NMJ. NGF binds to ErbB receptors and activates the phosphotidylinsitol-3 kinase (PI3K) pathway. Similar to BDNF and IGF-1, increased NGF expression activates the AKT pathway to promote protein synthesis. A 3 day treatment of old rats with exogenous NGF (40 nM) increased total protein synthesis in diaphragm by 29% and AKT phosphorylation by 30 fold compared to untreated muscle. This study suggests that NGF contributes to the protein synthesis process (Hellyer et al., 2006). A deficiency in NGF leads to muscle atrophy. Transgenic mice that expressed antibodies against NGF have muscle fibers with central nuclei, vacuoles in the mycoplasma and infiltratory lymphocytes cells which is a sign for muscle atrophy (Capsoni et al., 2000).

**MUSCLE PLASTICITY AND β2-ADRENERGIC RECEPTORS**

Epinephrine and norepinephrine are catecholamines that are released by an adrenal gland during stress or muscle activity. Norepinephrine is also released by sympathetic neurons that
innervate skeletal muscle. Catecholamines bind to α or β adrenergic receptors and elicit their response through secondary intracellular signaling pathways. The primary locations of α-adrenergic receptors are the smooth muscle cells of blood vessels, which express two isoforms, α₁ and α₂, to induce vasoconstriction. β-adrenergic receptors β₁, β₂ and β₃ are also expressed and cause vasodilation during exercise (Lynch & Ryall, 2008). This final response is a function of the type and concentrations of the catecholamines. Low concentration and especially adrenaline during exercise selectively binds to β₂-adrenergic receptors causing vasodilation to increase blood flow and oxygen delivery to active muscles (Lynch & Ryall, 2008). At high concentrations, catecholamines, especially noradrenaline, bind to α₁-adrenergic receptors to initiate vasoconstriction such as during hemorrhage. Furthermore, catecholamines play a role in modulating muscle metabolism such as promoting muscle glycogen breakdown during exercise to produce more ATP. Even in the absence of exercise, an acute catecholamine treatment in rat gastrocnemius and plantaris muscles reduces the glycogen level by 24-48%, while glucose and glucose-6-phosphate content increases by 51% and 75%, respectively (Clausen et al., 2011). Finally, catecholamine also plays a role in maintaining membrane excitability during exercise by activation of Na⁺-K⁺ pumps (Clausen et al., 2011).

Activation of β₂-adrenoceptors during exercise also has long term effects on gene expression in skeletal muscles. It is believed to be involved in muscle growth, muscle development and repair after injury (Lynch & Ryall, 2008). β₂-adrenoceptors are coupled with G-stimulatory protein (Gs) and adenylyl cyclase to convert ATP to cAMP, a secondary messenger. cAMP then activates protein kinase A (PKA) which regulates several intracellular signaling pathways. PKA is a heterotetramer protein composed of two regulatory (R) and two catalytic (C) subunits. cAMP is required to bind to the R subunits to induce conformational changes in PKA causing the
dissociation of the R subunits from the C subunits. The resulting active C subunit phosphorylates various serine and threonine residues on targeted proteins to initiate a protein synthesis pathway such as the AKT signalling and an inhibition of degradation pathway such as the Fox pathway. Furthermore, the active C subunit can enter the nucleus via passive diffusion to initiate transcription by phosphorylating CRE, a transcription factor which is involved in myogenic gene expression to activate the MEF2 family of the transcription factors for a differentiation process (Lynch & Ryall, 2008).

Stimulation of skeletal muscle hypertrophy and inhibition of skeletal muscle atrophy is also regulated by β2-adrenergic receptors. Several β2-adrenergic agonists have been developed over the years such as salbutamol, clenbuterol, formoterol, fenoterol, terbutaline, and bambuterol (Lynch & Ryall, 2008). β2-agonists have the ability to increase skeletal muscle mass and decrease body fat. They are considered as potential therapeutic approaches for treatment of clinical defects such as nerve damage, spinal cord injury, denervated muscle, and myotoxic injury, which are all conditions associated with loss of muscle mass and strength (Zeman et al., 1987; Sillence et al., 1991; Hinkle et al., 2002). For example in innervated soleus, TA and gastrocnemius muscle, administration of clenbuterol (1.6 mg /kg body mass) for 5 weeks increases muscle mass by 17-64%, total protein content by 22-50% and CSA by 10-53% (Ingalls et al., 1996). It has similar effects on denervated muscle as well. Treating denervated muscles with clenbuterol for 2-3 weeks increases muscle protein content by 95–110% and muscle mass by 39–50% when compared to untreated denervated muscle (Zeman et al., 1987). Together these studies suggest that β2-agonists promote anabolic processes in skeletal muscle. Moreover, further studies have been done to confirm that the anabolic effect of stimulation of β2-adrenergic receptors is not related to β1-activation. Treatment with ICI118551, a selective β2-adrenergic receptor antagonist,
causes atrophy by itself and inhibits the anabolic effect of clenbuterol (Silence et al., 1991). Finally, clenbuterol still induces hypertrophy in innervated TA and gastrocnemius muscle and reverses the atrophy effect in denervated muscles in β1-adrenoeceptor knockout mice (Hinkle et al., 2002). All these studies provide evidence regarding the potential for β2-agonists to reverse muscle atrophy and to be useful for stimulating muscle growth in muscle wasting diseases.

Interestingly, administration of β2-agonists abolishes the effect of training. For example, after 8 weeks of running exercise, the total work performance of mice increased by 58% compared to untrained mice, but mice treated with clenbuterol only had a performance improvement by 25% (Ingalls et al., 1996). Another study found that the effects of strength training and clenbuterol combination on muscle hypertrophy were not additive in fast twitch muscle (Mounier et al., 2007).

**SKELETAL MUSCLE FIBER IN CULTURE**

Most studies on muscle plasticity are carried out in vivo. It is therefore not easy to separate the changes in fiber phenotype that strictly depend on the intrinsic properties of skeletal muscle versus those modulated by exogenous factors. Another approach used to study muscle plasticity is the use of precursor cells in an attempt to produce muscle fibers in vitro (Hellyer et al., 2006). This approach however does not produce fully differentiated and mature fibers (Pennisi et al., 2011). Single FDB adult muscle fibers can easily be separated by trituration following a collagenase digestion. These fibers can then be maintained in culture. Such an approach allows for a better control with regards to exposing fibers to endogenous factors. However, so far, most investigators have used such a preparations to study skeletal muscle myogenesis from satellite cells in vitro (Danoviz & Yablonda-Reuveni, 2012; Pasut et al., 2013)
or to study the mobility of extrajunctional acetylcholine receptors in denervated adult muscle fibers (Stya & Axelrod, 1984). Very few studies have actually documented the changes in muscle fiber characteristics (Bekoff & Betz, 1977b; Morel et al., 2010). Bekoff and Betz (1977) were the first to characterize such fiber preparations in culture. They found decreases in resting membrane potential from the normal -70/-80 mV range to -60 mV. Morel et al. (2010) reported an increase in Nav1.5 channels, the embryonic muscle Na\(^+\) channels, after 7 days in culture.

The major problem of using single muscle fibers in culture is perhaps the difficulty in maintaining them alive. Bischoff (1997) reported that only 1-2% of fibers were viable for 3 weeks (Bischoff, 1997). Plating fibers in Vitrogen gel improved viability by 38% but only for 96 h (Jay & Barald, 1985). There is therefore a need to develop a protocol to maintain adult single fibers in culture for longer periods of time in order to study muscle plasticity.

**OBJECTIVES OF THIS STUDY:**

Considering the potential advantages of studying muscle plasticity in culture and the need for maintaining muscle fibers for a long time in culture, the objectives of this study were: 1) to investigate the best approach to maintain muscle fibers in culture by measuring the morphological and physiological changes that occur when skeletal muscle fibers are incubated over a long periods of time; to determine whether 2) chronic stimulation and 3) catecholamines, reduce atrophy and improve contractility in muscle fibers maintained in culture as they do in vivo.
METHODS AND MATERIALS

ANIMALS

Experiments were carried out using single fibres from flexor digitorum brevis (FDB) muscles from CD-1 (Charles River, Canada). Mice were two to four months in age and weighed 20-30 g. Mice were fed ad libitum, and housed according to the guidelines of the Canadian Council for Animal Care (CCAC). The Animal Care Committee of the University of Ottawa approved all experimental procedures used in this study. Mice were anaesthetized with a single intraperitoneal injection of 2.2 mg ketamine, 0.44 mg xylazine and 0.22 mg acepromazine per 10 g of body mass. Subjects were then sacrificed by cervical dislocation.

SINGLE FIBRE PREPARATION

Single fibres were isolated from FDB bundles by a method involving a collagenase digestion as previously described (Selvin et al., 2014). Briefly, FDB muscles were incubated 3 h at 37°C in minimum essential medium (MEM) with Earle’s salt and L-glutamine (MEM, Gibco, Canada) supplemented with 10% heat inactivated foetal bovine serum (FBS, Gibco, Canada), 0.2% (w/v) collagenase type I (Worthington, USA), 100 units/ml of penicillin and 100 μg/ml of streptomycin (Gibco, Canada). Following incubation, fibres were separated by gentle trituration in three ml of collagenase-free culture medium. After being put on either glass coverslips or plastic culture dish (see below) fibres were incubated another 30 min to allow them to settle on the adhering substrate and become attached. Culture medium was then added to cover the entire coverslip and incubated at 37°C until used.

CULTURE CONDITIONS
Several different culture conditions were tested. Two culture medium were tested: i) Minimum essential medium with Earle’s salt and L-glutamine (MEM, Gibco, Canada) and ii) Dulbecco’s Modified Eagle Medium High Glucose (DMEM, Gibco, Canada) supplemented with 10% FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin (Gibco, Canada). With regard to plating, aliquots (100 μl) of concentrated fibre-containing medium were placed on either 12 mm diameter coverslips (VWR, Canada) or culture dish (Falcon, Canada) pre-coated with Matrigel (VWR, Canada), collagen alone or collagen/laminin mixture (Sigma, Canada). The frequency of changing 50% of the medium every day or every two days was also tested. The effect of inhibition of satellite cells proliferation on fiber survival by using 50 μM of Cytosine β-D-arabinofuranoside (Sigma, Canada) also was tested. Finally, the effect of chronic stimulation was tested. Contractions were elicited with 200 msec trains of 20 Hz or 50 msec trains of 80 Hz and 16 V every 15 min (IonOptix, U.S.A.).

**THRESHOLD AND MYOPLASMIC [Ca\(^{2+}\)] \(_{i}\) MEASUREMENT**

Coverslips containing single FDB fibres were mounted into a 370 μl chamber (model RC-25, Warner Instruments, USA). Fibers were continuously super-fused with a physiological solution at a rate of 3 ml/min. The control physiological solution contained (mM): 118.5 NaCl, 4.7 KCl, 2.4 CaCl2, 3.1 MgCl2, 25 NaHCO3, 2 NaH2PO4 and 5.5 D-glucose. All solutions were continuously bubbled with 95% O2–5% CO2 and had a pH of 7.4. Experimental temperature was 37°C and controlled by simultaneously heating the plate in which the chamber was mounted, and pre-heating the physiological solution, using a dual channel heater controller (model TC-344B, Warner Instruments, USA).

**Threshold measurement**
Fibres were elicited to contract by field stimulation, generated by two carbon electrodes running along each side of the culture dish wall (C-pace) and connected to a C-pace EP culture pacing system (Ion Optix, USA). Stimulations were 200 ms trains of 100 Hz 0.4 ms pulses at varying voltages.

[Ca^{2+}]_i measurement

Fibres were loaded with fura-2 (Ca^{2+} fluorescent indicator) by incubating fibres with 5 µM fura2-AM (Molecular Probes, Canada) for 30 min at 37°C similar to the protocol described by Selvin et al 2014. Fibers were elicited to contract with 200 ms trains of 0.4 ms 10 V pulses at different frequency. The coverslip containing the fibres was then transferred to the RC-25 experimental chamber. Fura-2 was excited alternatively between 340 nm and 380 nm and emission was measured at 510 nm. Light excitation and emission measurements were carried out using the IonOptix dual fluorescent contractility device (USA) containing the following filters: 340 ± 12 nm, 380 ± 6 nm and 505 ± 6 nm. Data acquisition was set at 200 Hz. The fluorescence ratio (R) was calculated by dividing the light emitted for two msec during the excitation of 340 nm (numerator) by the light emitted for two msec while the excitation was 380 nm (denominator). [Ca^{2+}]_i was then calculated as follows:

\[
[Ca^{2+}] = K_d \cdot \beta \left( R - \frac{R_{\text{min}}}{R_{\text{max}}} - R \right) \quad (1)
\]

where Kd is the dissociation constant, which is 224 nM at 37°C (Li et al., 2008); β is the fluorescence ratio at 380 nm excitation of Ca^{2+} free divided by Ca^{2+} bound fura-2. RMIN is the minimum fluorescent ratio when all fura-2 are Ca^{2+} free. RMIN was measured by exposing fibers to a physiological saline solution with zero Ca^{2+} to which was added 100 µM ionomycin, a Ca^{2+} ionophore, and 5 mM EGTA. RMAX is the maximum fluorescent ratio obtained when all fura-2 are Ca^{2+} bound. RMAX was measured by exposing fibers to a physiological solution to which was
added 100 µM ionomycin and 15 µM Ca\(^{2+}\) (unbuffered). It was not possible to measure \(R_{\text{MIN}}\) and \(R_{\text{MAX}}\) for each fiber because \(R_{\text{MIN}}\) had to be measured first and had the subsequent measurement \(R_{\text{MAX}}\) resulted in a range of values that were less that was less than the range measured without a preceding \(R_{\text{MIN}}\) measurement (data not shown). The variability in \(R_{\text{MIN}}\) was small, ranging from 0.3 to 0.7, whereas the variability in \(R_{\text{MAX}}\) was very large from 1.6 to 5.5. When measured ratios during contractions were carried out, it was realized that at a constant \(R_{\text{MAX}}\) the calculated resting \([\text{Ca}^{2+}]_i\) from the lowest and highest \(R_{\text{MIN}}\) values gave rise to very small differences. For example for a resting ratio of 0.562, the calculated resting \([\text{Ca}^{2+}]_i\) ranged from 0.071 to 0.037 µM. Similarly, from a fura-2 ratio during contraction of 1.81, the calculated \([\text{Ca}^{2+}]_i\) ranged from 0.92 to 0.85 µM. So, each day a series of experiments were carried out, one fiber was used to measured \(R_{\text{MIN}}\) and a mean value of 0.505 ± 0.029 was used for the \([\text{Ca}^{2+}]_i\) calculations. The situation was very different for \(R_{\text{MAX}}\). For example, the calculated \([\text{Ca}^{2+}]_i\) during contraction from a ratio of 1.93 using the mean \(R_{\text{MIN}}\) and a \(R_{\text{MAX}}\) of 0.505 and 3.6 give rise to 0.6 µM \(\text{Ca}^{2+}\), while for a \(R_{\text{MAX}}\) of 2.926 the calculated \([\text{Ca}^{2+}]_i\) was 1.2 µM. So, \(R_{\text{MAX}}\) was measured in each tested fibers and the \([\text{Ca}^{2+}]_i\) were calculated using the specific \(R_{\text{MAX}}\) value for each fiber.

**STATISTICAL ANALYSIS**

ANOVA was used to determine statistically significant differences. Split plot designs were used because fibres from one mouse were tested at all times in culture or experimental times. ANOVA calculations were made using the Version 9.0 GLM (General Linear Model) procedures of the Statistical Analysis Software (SAS Institute Inc., Cary, NC USA). When a main effect or an interaction was significant, the least square difference (L.S.D.) was used to locate the significant
differences (Steel & Torrie 1980). The word “significant” refers only to a statistical difference (P < 0.05).
RESULTS

MORPHOLOGY CHANGES OF SKELETAL MUSCLE FIBERS IN CULTURE

In a first series of experiments, single FDB fibers were separated by trituration following a collagenase digestion in Minimum Essential Medium (MEM) containing 10% Fetal Bovine Serum (FBS, V/V). They were then plated on glass coverslip coated with Basement Membrane Matrix (Matrigel) to increase fiber adherence. Coverslip where then put in culture dish and 50% of the medium was replaced with fresh medium every day. After trituration, fibers had a cylindrical shape with multiple nuclei and satellite cells located along the fiber surface (Fig.1A). The striation of A bands (dark bands) and I bands (light band) were clearly visible. This morphology was called a normal morphology. Morphology changed drastically while fibers were being maintained in culture for several days at 37°C. Evidence of degenerating fibers was observed when they lost their normal shape (Fig.1C) or contained vacuoles in the cytoplasm (Fig.1B). Finally, fibers were supercontracted when fiber length decreased from a normal length (Fig.1D).

The number of fibers with normal morphology, that were degenerating or had supercontracted were counted after being maintained in culture for several days. It was also noted that as culture medium was changed, fibers were lost as they floated away from coverslip. So, the mean number of counted fibers per mice was also calculated. However, to avoid prolonged and numerous exposures of fibers outside the incubator, a count was only preformed once per well containing coverslips. Effort was made to have the same numbers of fibers per coverslips, but this was not always achieved in some experiments. So, the mean total numbers of counted fibers per mouse are reported in each figure to indicate how many fibers were used to calculate the percent values of fibers with normal morphology, that were degenerating and supercontracted fibers.
Figure 1. Morphology of single FDB muscle fibers maintained in culture. A, Example of a normal fiber with straight appearance and clear A and I bands. Example of degenerating fibers observed after 4th day which are B) have lost their fiber integrity or C) partially contracted and containing vacuoles. D) Example of supercontracted (red arrow) fibers occurring after 3 day in culture. White arrows in D shows the presence of mono-nucleated cells.
During the first 72 hours of incubation, most fibers (84-92%) had normal morphology (Fig. 2A). The remaining fibers were supercontracted (Fig. 2C) as no degenerating fibers were observed (Fig. 2B). By day 4, the number of normal fibers had significantly decreased to 72% and the remaining fibers being supercontracted (34%). By day 5, the number of normal fibers was 45% while the remaining fibers were degenerating fibers (22%) and supercontracted fibers (32%). Finally, no fibers were present on coverslips by day 6 (Fig. 2D).

So, to study skeletal muscle plasticity in culture, a protocol had to be developed to improve fiber survival. A series of experiments were therefore conducted to investigate different adhering surfaces (glass coverslip vs. plastic culture plate), adhering substances (collagen/laminin combination vs. Matrigel), the composition of the culture media and how often the culture medium had to be changed. Due to the large number of conditions to test, conditions could not be tested all at once from one preparation; i.e., one mouse. So, different combinations of conditions were tested from each single fiber preparation obtained from a mouse. Then, for a given set of experimental conditions data were pooled together. As a consequence of this approach, some data for a given set of conditions appears in more than one figure.

**Adhering surface and frequency of culture medium solution changed**

Glass coverslips are normally used for the measurements of myoplasmic calcium concentration ([Ca^{2+}]_i) with fura-2. However, Matrigel adheres better on plastic culture dish than on glass coverslip. To test whether the decrease in the number of fibers with normal morphology was affected by how well Matrigel adheres to glass coverslip, the changes in morphology was also determine using plastic culture dish, a more conventional method by which any cell are kept in culture. The frequency of changing 50% of the medium every day or every two days was also tested. When culture media was changed every day, there was no significant difference about how
Figure 2. Fibers with normal morphology were completely gone by day 6 in culture. Fibers were cultured in MEM supplemented with 10% FBS. Fibers were plated on Matrigel coated glass coverslip. 50% of culture medium solution was changed every day. A) Number of fibers with normal morphology. B) Number of degenerating fibers. C) Number of supercontracted fibers. Numbers of fibers are expressed as a percent of the total number of fibers present on coverslip. D) Mean total number of counted fibers per mice. Vertical bars represent the S.E. of 10 mice.

§ Indicate when the mean value became significantly different from 100% in A or 0% in B and C; ANOVA, least significant difference (LSD), P < 0.05.
the number of fibers with normal morphology decreased over time between plastic culture dish and glass coverslip, except at day 4 the number of fibers with normal morphology had significantly dropped to 86% in plastic dish compared to 72% in glass coverslips (Fig. 3A). By day 6, there was no fibers left with glass coverslip as well as plastic dish (Fig. 3D). The numbers of degenerating and supercontracted fibers were also not different between the adhering surfaces (Fig. 3B, C). Experiments were repeated in which Matrigel was replaced by collagen, and there were still no differences between the two adhering substances (Appendix I).

When half the medium was changed every 2 days, significant differences were observed between plastic dish and glass coverslips. The number of fibers with normal morphology was significantly greater at day 5 with glass coverslip, which contained 66% of normal fibers compared to 38% with culture dish (Fig. 3E). By the 8th day the number of normal fibers was significantly decreased to 41% with glass coverslip compared to none for plastic dish. Furthermore, with glass coverslip, fibers were still present up to 21 days, albeit none looked normal as most were degenerating fibers (72%) and others supercontracted (27%) (Fig. 3F, G).

So, while the improvement in terms of keeping fibers with normal morphology was small, the capacity of prolonging the presence of fibers in culture from 5 to 21 days was significant because the next step was to find conditions that reduce the degenerating process.

**Inhibition of satellite cells proliferation and MEM vs. DMEM culture medium**

As shown in Fig.1D, several mononucleated cells appeared over time as muscle fibers degenerated or supercontracted. These cells were most likely satellite cells (Tavi et al., 2010) and/or fibroblasts. The question then was whether these cells are contributing to fiber degeneration.
Figure 3. Fiber survival was improved when plated on glass coverslip and culture media changed every two days. Fibers were cultured in MEM supplemented with 10% FBS. Glass coverslip and plastic culture dish were covered with Matrigel. 50% of culture medium solution was changed every day (A-D) or every 2 days (E-H). A, E) Number of fibers with normal morphology. B, F) Number of degenerating fibers. C, G) Number of supercontracted fibers. Numbers of fibers are expressed as a percent of total number of fibers present on coverslip the day they were counted. D, H) Mean total number of counted fibers per mice. Vertical bars represent the S.E. of 2-8 mice. § Indicate when the mean value became significantly different from 100% in A or 0% in B and C. † Mean value from glass coverslip was significantly different from mean value in plastic dish. * Mean value from a change in culture medium every two days was significantly different from the change every day; ANOVA, and LSD, P < 0.05.
Cytosine β-D-arabinofuranoside (AraC) is a mitotic blocker that selectively inhibits DNA synthesis but not RNA synthesis and is often used to prevent proliferation of satellite cells (Troy et al., 2012).

Also, Minimum Essential Medium (MEM) and Dulbecco’s Modified Eagle Medium (DMEM) are the two most commonly used culture media for experiments involving single muscle fibers (Shefer & Yablonka-Reuveni, 2005; Robison et al., 2011; Starkey et al., 2011). The difference between MEM and DMEM is that the latter contains additional amino acids and vitamins (Appendix IV). Although it had been demonstrated that a collagenase digestion in MEM yields better single fibers than in DMEM (Selvin et al., 2014), the possibility that the presence of more nutrients in DMEM improves fiber survival under culture conditions was tested following the collagenase digestion in MEM.

In MEM medium, the addition of AraC significantly improved fiber survival (Fig. 4A). By day 14 and 17, there were still 45-48% of fibers with normal appearance in the presence of AraC whereas there was none in its absence. Even by 21 days, 17% of fibers appeared normal. In DMEM medium, AraC also significantly improved fiber survival (Fig. 4E). There was no normal fiber by day 5 in the absence of AraC whereas from day 8 to 21 days, 22-36% of the fibers had normal appearance. Another impact of AraC was significantly disappearance of degenerating fibers in both MEM and DMEM (Fig. 4B, F). There was, however, a significant increase in the number of supercontracted fibers, especially in DMEM (Fig. 4C, G).

Difference between MEM and DMEM were also observed. In the absence of AraC, the number of normal fibers significantly dropped to almost zero by day 5 in DMEM whereas these were still 41% of normal fibers at day 8 in MEM. In the presence of AraC, MEM was still the best
Figure 4. Fiber survival was better in MEM than DMEM and improved when AraC, a cell mitosis inhibitor, was added. All collagenase digestions were carried out in MEM and following trituration fibers were incubated with either MEM (A-D) or DMEM (E-H) supplemented with 10% FBS in the absence or presence of 50 µM AraC. Fibers were plated on glass coverslip coated with Matrigel. 50% of culture medium was changed every 2 days. A, D) Number of fibers with normal morphology. B, E) Number of degenerating fibers. C, F) Number of supercontracted fibers. G, H) Mean total number of counted fibers per mice. Vertical bars represent the S.E. of 7-5 mice. Indicate when the mean value became significantly different from 100% in A or 0% in B and C. † Mean value from using AraC was significantly different from mean value of no AraC. * Mean value from MEM was significantly different from mean value of DMEM; ANOVA, and LSD, P < 0.05.
medium, the major difference being observed between days 8 to 17 where in MEM 45-58% of the fibers had normal appearance versus only 24-36% in DMEM (Fig. 4 A, E).

**Adhering substrate**

Matrigel contains laminin and collagen IV as well as several factors that promote cell growth such as Heparan sulfate proteoglycans, Entactin/Nidogen, TGF-β (transforming growth factor β) and Insulin-like growth factors. However, the use of Matrigel will interfere with studies on the importance of various myokines and neurotrophic factors on muscle plasticity. A better approach would be to use collagen and laminin as the only adhering substances to allow for full control of which specific factors fibers are exposed to. So, experiments were carried out to document the extent of fiber survival comparing Matrigel and collagen/laminin as adhering substances. These experiments have also been done with and without addition of AraC.

In the absence of AraC, the decrease in the number of normal fibers was significantly slower with Matrigel than with collagen/laminin (Fig.5A). For example, by day 3, 81% of fibers had normal morphology with Matrigel whereas with collagen/laminin the value had significantly dropped to 41%. Then at day 8, 41% of the fibers had a normal morphology with Matrigel compared to none with collagen/laminin. The number of degenerating fibers in collagen/laminin had a peak of 46% at day 5 (Fig.5B) while supercontracted fibers were observed on the first 5 days only (Fig.5C).

In the presence of AraC, on the other hand, collagen/laminin appeared significantly better than Matrigel (Fig. 5E). Significant differences were observed between day 8 and day 17 as the number of fibers with normal morphology averaged between 62% and 73% with collagen/laminin
Figure 5. In the presence of 50 µM AraC, fiber survival was slightly better with collagen/laminin than with Matrigel, while the reverse was observed in the absence of AraC. Fibers were kept in MEM supplemented with 10% FBS without (A-D) or with (E-H) 50 µM AraC. Fibers were plated on glass coverslips coated with either Matrigel or collagen/laminin. 50% of culture media were changed every 2 days. A, E) Number of fibers with normal morphology. B, F) Number of degenerating fibers and C, G) supercontracted fibers. D, G) Mean total number of counted fibers per mice. Vertical bars represent the S.E. of 2-7 mice. § Indicate when the mean value became significantly different from 100% in A or 0% in B and C. † Mean value from Matrigel was significantly different from mean value of collagen/laminin.

* Mean value from using AraC was significantly different from mean value of no AraC; ANOVA, and LSD, P < 0.05.
compared to 45% and 58% with Matrigel. While there was no difference in the number of degenerating fiber (Fig. 5F), there were less supercontracted fibers between 8 and 17 days when collagen/laminin was used (Fig. 5G). Similar experiments had also been carried out in which plastic dish was used instead of glass coverslips covered with Collagen; the results were similar in that AraC improved fiber survival (appendix II).

In summary, when FDB single muscle fibers were kept at rest in culture for several days, MEM was a better medium than DMEM. Fiber survival was improved by the addition of AraC to inhibit satellite cell or fibroblast mitosis. In the presence of AraC collagen/laminin was a slightly better adhering substrate than Matrigel. Finally, changing 50% of the culture medium every 2 days improved fibers survival as oppose to changing every day.

EFFECT OF CHRONIC STIMULATION ON FIBERS MORPHOLOGY

As discussed in the Introduction, chronic stimulation reverses the atrophy process of denervated muscle in situ. Considering that muscle fibers kept in culture behave like denervated fibers, the second objective is to determine if chronic stimulation also improves fiber survival in culture.

In the first series of experiments, fibers were stimulated at 80 Hz for 50 ms every 15 min. Notably, fibers survival was significantly worsened with chronic stimulation. After one day of stimulation 91% of stimulated fibers had normal morphology, which was not significantly different from fibers kept at rest (Fig.6A). By day 5, only 7% of the stimulated fibers had normal morphology compared to 68% for unstimulated fibers. Finally by 8 days, there were no fibers left with normal morphology compared to 19% at day 21 for resting fibers. There was no
Figure 6. Chronic stimulation worsened fiber survival in culture. MEM was supplemented with 10% FBS and 50 µM AraC. Fibers were plated on glass coverslip coated with collagen/laminin. 50% of culture medium was changed every 2 days. Fibers were stimulated with 50 msec train of pulses at 80 Hz every 15 min. A) Number of fibers with normal morphology. B) Number of degenerating fibers. C) Number of supercontracted fibers. D) Mean total number of counted fibers per mice. Vertical bars represent the S.E. of 4-5 mice.

§ Indicates when the mean value became significantly different from 100% in A or 0% in B and C.

† Mean value from resting fibers was significantly different from mean value of stimulated fibers; ANOVA, and LSD, P < 0.05.
degenerating fibers when fibers were chronically stimulated (Fig.6B). Instead, by day 5 all fibers had supercontracted (Fig. 6C).

**Importance of insulin and taurine**

Insulin is known to increase glucose uptake, glycolysis, and oxidative phosphorylation which help maintained good energy balance. It also increases amino acid transport and protein synthesis which may help in reversing the atrophy process. Finally, it also activates Na\(^+\)-K\(^+\) pumps, an affect that can hyperpolarise the cell membrane. Taurine is an amino acid known to stabilize resting membrane potential (Qi *et al.*, 1995). If the supercontractions are due to either poor energy balance or maintenance of normal resting potential, then the addition of insulin and taurine may improve fiber survival when fibers are chronically stimulated. The insulin was tested at 150 µU/ml, which is an activity level that increases glucose uptake by 60% in EDL and soleus muscle (Miki *et al.*, 2002). Taurine was tested at 1 mM, which is higher than in the plasma 44 µM (E A Trautwein, 1990).

Two stimulation protocols were used: i) 100 ms train at 20 Hz every 15 min, which mimics endurance training, and ii) 50 ms train at 80 Hz every 15 min, which mimics strength training. Overall, the results showed no significant impact on fiber survival between conditions and stimulation pattern (Fig.7). If the addition of insulin and/or taurine helps in preserving resting membrane potential in normal fibers, then it should have an impact on the threshold for contraction. To test this, the number of contracting fibers at different stimulus strength were counted. For control condition, at day 0 the minimum voltage required to trigger contraction at 20 Hz was 4 V as 2% of the fibers contracted (Fig. 8A). Half the fibers (V\(_{50}\)) were contracting at 12 V and all of them at 22 V. On day 1, there was a shift in the number of contracting fibers-stimulus strength relationship toward less voltage as half the fibers were now contracting at 8 V. By day 3,
**Figure 7.** Neither insulin nor taurine significantly improved fiber survival during chronic stimulation. Fibers were chronically stimulated with 100 ms long train of pulses at 20 Hz for 100 ms every 15 min (A-C) or with 50 ms long train of pulses at 80 Hz every 15 min (D-F). MEM was supplemented with 10% FBS, 50 µM AraC, 150 µU/ml insulin and 1 mM taurine. Fibers were plated on glass coverslip coated with collagen/laminin, 50% of medium were changed every 2 days. A, D) Number of fibers normal morphology. B, E) Number of supercontracted fibers. E, F) Mean total number of counted fibers per mice. Vertical bars represent the S.E. of 4-2 mice. § Indicate when the mean value became significantly different from 100% in A and D or 0% in B and E; ANOVA, and LSD, P < 0.05.
Figure 8. The number of contracting fibers-stimulus strength relationship was shifted to higher strength in chronically stimulated fibers at 20 Hz with significant improvement with insulin or taurine on day 3 when tested individually but not in combination. In culture, fibers were stimulated with 100 msec long train of pulses at 20 Hz every 15 min. MEM was supplemented with 10% FBS, 50 µM AraC, 150 µU/ml insulin and/ or 1 mM taurine. Fibers were plated on glass coverslip coated with collagen/laminin, 50 % of medium were changed every 2 days. To count the number of contracting fibers, contractions were elicited with 200 msec long trains of pulses at 100 Hz at the indicated voltages. Vertical bars represent the S.E. of 4-2 mice.

† Mean value from number of contracting fibers was significant different from previous day ANOVA, and LSD, P < 0.05.
Figure 9. The stimulus strength–number of contracting fibers relationship was shifted to higher strength in chronically stimulated fibers at 80 Hz with no significant improvement with the addition of insulin or taurine. Fibers were chronically stimulated with 50 ms long train of pulses at 80 Hz every 15 min. MEM was supplemented with 10% FBS, 50 µM AraC, 150 µU/ml insulin and/or 1 mM taurine. Fibers were plated on glass coverslip coated with collagen/laminin, 50 % of medium were changed every 2 days. To count the number of contracting fibers, contractions were elicited with 200 msec long trains of pulses at 100 Hz at the indicated voltages. Vertical bars represent the S.E. of 4-2 mice.

† Mean value from number of contracting fibers was significant different from previous day ANOVA, and LSD, P < 0.05.
no fibers were contracting. Insulin improved contractility at days 1 and 3 (Fig. 8B). Taurine also improved contractility at day 3 (Fig. 8C), but the lowest voltage that triggered some fibers to contract was much greater with taurine than with insulin. Adding both insulin and taurine did not improve contractility when compared to their absence (Fig. 8A, D). Contractile improvement by insulin and taurine was almost nonexistent when the stimulation frequency in culture was increased to 80 Hz (Fig. 9).

**Inhibition of satellite cells or fibroblasts proliferation when fibers were chronically stimulated**

Inhibition of satellite cell or fibroblasts proliferation with AraC improved fibers survival kept at rest, but it remained to be determined if the same occurs with chronic stimulation. The experiments were also carried out using Matrigel or collagen/laminin as the adhering substrate in order to determine whether or not some of the factors in Matrigel are important when fibers are chronically stimulated.

When collagen/laminin was used as the adhering substrate, AraC worsened survival of chronically stimulated fibers (Fig. 10A). By day 5, the number of fibers with normal morphology was 7% when AraC was present, whereas in the absence of AraC the numbers of fibers with normal morphology were 60% and 25% after 5 and 8 days, respectively. Even in the absence of AraC, degenerating fibers were not observed (data not shown). The absence of AraC significantly delayed the time when all fibers supercontracted (Fig. 10B). In Matrigel, AraC also worsened survival of chronically stimulate fibers. The largest difference was at day 3 when 45% of fibers had normal morphology in the absence of AraC compared to 19% with AraC (Fig. 10D). Again, the absence of AraC appeared to delay supercontraction (Fig. 10E). With chronic stimulation, fiber survival was better with collagen/laminin than with Matrigel. In the presence of AraC, a significant difference was observed at 3 days where 53% of fibers plated on collagen/laminin
Figure 10. AraC worsened fiber loss when chronically stimulated. MEM was supplemented with 10% FBS with or without 50 μM AraC. Fibers were plated on glass coverslip coated with either Matrigel (A-C) or collagen/laminin (D-F). 50% of medium was changed every 2 days. Fibers were stimulated in culture with 50 msec train of pulses at 80 Hz every 15 min. Under those conditions degenerating fibers were not observed. A, D) Number of fibers with normal morphology. B, E) Number of supercontracted fibers. C, F) Means total number of counted fibers per mice. Vertical bars represent the S.E. of 4 mice. § Indicate when the mean value became significantly different from 100% in A and D or 0% in B and E. † Mean value from no AraC was significantly different from mean value of using AraC. * Mean value from collagen/laminin was significantly different from mean value of Matrigel; ANOVA, and LSD, P < 0.05.
have a normal morphology (Fig. 10A) compared to only 19% when plated on Matrigel (Fig. 10D). However, by day 5 all fibers were supercontracted regardless of the adhering substrate. In the absence of AraC, the numbers of normal fibers in collagen/laminin were 60% and 25% at day 5 and 8 respectively; these numbers were significantly greater than those for Matrigel as the numbers were respectively 15% and 3%. Some experiments were also conducted when fibers were stimulated at 20 Hz train 200 ms every 15 min using collagen and Matrigel coated either on glass coverslip or on plastic dish without AraC. Regardless of the adhering substance or adhering surface, there was no improvement in fiber survival (Appendix III).

EFFECTS OF $\beta_2$ ADRENERGIC AGONIST ON FIBER MORPHOLOGY

As discussed in the introduction $\beta_2$-adrenergic receptor activation is another factor that improves in situ contractility function of denervated muscles (Lynch & Ryall, 2008). So, the third objective of this study was to determine if salbutamol, a $\beta_2$-adrenergic agonist, can improve fiber survival under culture conditions. For these experiments, fibers were divided into two groups: i) resting fibers exposed to AraC and ii) chronic stimulation (80 Hz for 50 ms every 15 min) with no AraC. Each of the rest and stimulation fiber groups were then subdivided into three groups: i) control; ii) 5 µM salbutamol, $\beta_2$-adrenergic agonist and iii) 0.1% (v/v) DMSO, which is the vehicle for salbutamol. Although insulin had no major impact it was kept to all culture media at a level similar to that in situ to ensure proper glucose uptake.

Under resting condition, salbutamol did not improve fiber survival. For the first 5 days, there was no significant difference in the number of normal fibers between control, DMSO and salbutamol conditions (Fig. 11A). Then by day 14, the decreases were significantly greater in salbutamol as the number of normal fibers was 17% compared to 67% and 62% for control and
Figure 11. Salbutamol worsened survival of resting fibers. MEM supplemented with 10% FBS and 50 μM AraC. MEM was also supplemented with 300 μU/ml insulin. For salbutamol and DMSO condition, the concentration was 5 μM salbutamol and 0.1% (v/v) DMSO, respectively. 50% of culture medium was changed every 2 days. Fibers were plated on glass coverslip coated with collagen/laminin. A) Number of fibers with normal morphology. B) Number of degenerating fibers. C) Number of supercontracted fibers. D) Mean total number of counted fibers per mice. Vertical bars represent the S.E. of 5 mice.

§ Indicate when the mean value became significantly different from 100% in A or 0% in B and C.

† Mean value from control was significantly different from mean value of salbutamol and DMSO; ANOVA, and LSD, P < 0.05.
Figure 12. Salbutamol worsened fibers survival during chronic stimulation. MEM was supplemented with 10% FBS and 300μU/ml insulin. Salbutamol and DMSO concentrations were 5 μM and 0.1% (v/v), respectively. Fibers were plated on glass coverslip coated with collagen/laminin. 50% of medium was changed every 2 days. In culture, fibers were stimulated with 50 msec train of pulses at 80 Hz every 15 min. A) Number of fibers with normal morphology. B) Number of supercontracted fibers. C) Mean total number of counted fibers per mice. Vertical bars represent the S.E. of 5 mice.

§ Indicate when the mean value became significantly different from 100% in A or 0% in B.

† Mean value from control was significantly different from mean value of salbutamol and DMSO; ANOVA, and LSD, P < 0.05.
DMSO exposed fibers, respectively. Salbutamol had no significant effect on the number of degenerating fibers, except at day 14 and 17 (Fig. 11B), while it significantly increased the number of supercontracted fibers (Fig. 11C). From day 8 to 14, the number of supercontracted fibers significantly increased from 13% to 72% with salbutamol, while with DMSO the increase was only from 14% to 32% and for control fibers from 26% to 33% (Fig. 11C).

Under chronic stimulation, salbutamol also worsened fiber survival. After one day of stimulation, 96-100% of fibers had normal morphology and around 3-4% of fibers were supercontracted for all these conditions (Fig. 12A). By day 5, 60% of control fibers had normal morphology compared to only 21-29% for fibers exposed to DMSO or salbutamol (Fig. 12A). By day 8, DMSO and salbutamol exposed fibers were supercontracted (97-100%) compared to only 74% for control fibers (Fig. 12B).

EFFECT OF CHRONIC STIMULATION AND SALBUTAMOL ON CONTRACTILE CHARACTERISTICS

In a last series of experiments, the effects of chronic stimulation and salbutamol on contractile characteristics were determined. Two parameters were measured: i) the voltage strength vs. the number of contracting fibers to determine fiber excitability and ii) the myoplasmic \([Ca^{2+}]_i\). (\([Ca^{2+}]_i\)).

Voltage strength

On day 0, the minimum stimulation strength to trigger contraction in some fibers was 4 V with half the fibers contracting at 10 V and all fibers contracting by 20 V (Fig.13A). After 7 days under resting condition, the number of contracting fibers stimulation strength relationship was significantly shifted toward higher strength. The minimum strength significantly increased from 4
to 8 V, $V_{50}$ significantly increased from 10 to 22 V and the maximum strength from 20 to 40 V. Further significant shifts toward higher strength occurred by day 14 as the minimum strength was 18 V and at 40 V only 40% of fibers were contracting. By contrast, chronic stimulation significantly induced a shift in the number of contracted fibers-stimulus strength relationship toward lower stimulus strength (Fig. 13B). At day 5, the minimum strength had significantly decreased from 4 to 2 V, $V_{50}$ from 14 to 8 V and the maximum strength from 28 to 18 V. A further shift was observed from day 5 to day 8.

For resting fibers, both salbutamol and DMSO significantly worsened the stimulus strength required to trigger contraction. At day 7, the minimum voltage to trigger contraction was 24 V for DMSO and salbutamol compared to 8 V for control (Fig. 14A). Furthermore, only 33-49% of DMSO and salbutamol exposed fibers contracted at 40V compared to 100% for control. After 14 days, the number of contracted fibers-stimulus strength relationship was relatively similar to control fibers while a smaller shift was observed for DMSO exposed fibers (Fig. 14B).

On the other hand, salbutamol improved the number of contracting fibers stimulus strength relationship of chronically stimulated fibers. After 3 days of chronic stimulation, salbutamol caused a non-significant shift toward low stimulus strength, a shift that was even greater with DMSO (Fig. 15A). The $V_{50}$ for control, DMSO and salbutamol exposed fibers were 14, 12 and 14 V, respectively. After 5 days of chronic stimulation, the relationship was significantly shifted toward lower strength with both DMSO and salbutamol as the $V_{50}$ were 6 V compared to 8 V for control fibers (Fig. 15B).

**Myoplasmic $Ca^{2+}$**

On day 0, tetanic $[Ca^{2+}]_i$, defined as the $[Ca^{2+}]_i$ during a contraction increased as the
Figure 13. The stimulus strength–number of contracting fibers was shifted to higher strength in resting fibers while chronic stimulation caused a shift toward lower strength. MEM was supplemented with 10% FBS and 300 µU/ml insulin. Fibers were plated on glass coverslip coated with collagen/laminin. For resting fibers (A) MEM was also supplemented with 50µM AraC. 50% of culture medium was changed every 2 days. For chronically stimulated fibers (B), stimulation were 50 ms long train of pulses at 80 Hz every 15 min. For the measurements of contracting fibers at various voltages, contractions were elicited with 200 msec long train of pulses at 100 Hz and at the indicated voltages. Numbers of contracted fibers were expressed as a percent of total number of fibers present on coverslip the day they were counted. Vertical bars represent the S.E. of 5 mice.

† Indicate when the mean value became significantly different from previous day; ANOVA, and LSD, P < 0.05.
Figure 14. For resting fibers salbutamol and DMSO significantly shifted the number of contracting fibers-stimulus strength relationship at day 7 toward higher stimulus strength while only DMSO did the same at day 14. MEM was supplemented with 10% FBS, 300 µU/ml insulin and 50 µM AraC. Salbutamol and DMSO concentrations were respectively 5 µM and 0.1% (v/v). 50% of culture medium was changed every 2 days. Fibers were plated on glass coverslip coated with collagen/laminin. For the measurements of contracting fibers at various voltages, contractions were elicited with 200 msec long train of pulses at 100 Hz and at the indicated voltages. Numbers of contracting fibers were expressed as a percent of the total number of fibers present on coverslips. Vertical bars represent the S.E. of 5 mice.

† Indicate when the mean value became significantly different from day 7.

* Mean value from control was significantly different from mean value of salbutamol and DMSO; ANOVA, and LSD, P < 0.05.
Figure 15. Under chronic stimulation there was significant difference in stimulus strength-number of contracted fibers relationship between control, salbutamol and DMSO conditions. MEM was supplemented with 10% FBS, 300 µU/ml insulin and 50 µM AraC. Salbutamol and DMSO concentrations were respectively 5 µM and 0.1% (v/v). 50% of culture medium was changed every 2 days. Fibers were plated on glass coverslip coated with collagen/laminin. In culture, fibers were stimulated with 50 ms long train of pulses at 80 Hz every 15 min. Numbers of contracting fibers were expressed as a percent of the total number of fibers present on coverslips. For the measurements of contracting fibers at various voltages, contractions were elicited with 200 msec long train of pulses at 100 Hz and at the indicated voltages. Vertical bars represent the S.E. of 5 mice.

† Indicate when the mean value became significantly different from day 3.

* Mean value from control was significantly different from mean value of salbutamol and DMSO; ANOVA, and LSD, P < 0.05.
stimulation frequencies from 0.49 µM at 30 Hz to 1.27 µM at 100 Hz (Fig. 16A). Further increase to 120 Hz, however, decreased tetanic [Ca^{2+}]_i to 0.85 µM. Tetanic [Ca^{2+}]_i was reduced at all stimulation frequencies after 7 days in culture under resting conditions. The highest tetanic [Ca^{2+}]_i was only 0.4 µM at 120 Hz. Chronic stimulation did not improve tetanic [Ca^{2+}]_i. Tetanic [Ca^{2+}]_i after 3 days of chronic stimulation were similar to those that had been resting for 7 days (Fig. 16B). Another 5 and 8 days of chronic stimulation further decreased the tetanic [Ca^{2+}]_i so that by 8 days there were lower than for those resting for 7 days. Neither DMSO nor salbutamol affected tetanic [Ca^{2+}]_i in fibers kept under resting condition for 7 days (Fig. 17A). By day 14, tetanic [Ca^{2+}]_i was greater with salbutamol compared to control fibers but the effect was not significant (Fig. 17B). DMSO on the other hand, increased tetanic [Ca^{2+}]_i at 100-120 Hz.

Under chronic stimulation for 3 days, tetanic [Ca^{2+}]_i was significantly reduced in DMSO and salbutamol exposed fibers compared to control fibers. For example, at 120 Hz tetanic [Ca^{2+}]_i was 0.2 µM in the DMSO and salbutamol exposed fibers compared to 0.6 µM for control fibers (Fig. 18A). For day 5, there was a small improvement in tetanic [Ca^{2+}]_i with salbutamol between 30 and 50 Hz, and a significant improvement with DMSO between 50 and 120 Hz (Fig. 18B).
Figure 16. Chronic stimulation did not improve tetanic [Ca$^{2+}$]$_i$. MEM was supplemented with 10% FBS and 300 µU/ml insulin. For resting fibers, MEM was also supplemented with 50µM AraC. 50% of culture medium was changed every 2 days. Fibers were plated on glass coverslip coated with collagen/laminin. Stimulation in culture was 50 ms long train of pulses at 80 Hz every 15 min. Tetanic [Ca$^{2+}$]$_i$ defined as [Ca$^{2+}$]$_i$ the during contraction was recorded when fibers were stimulated with 200 ms train of 10 V pulses at indicated frequency. Vertical bars represent the S.E. of 5 mice.

† Indicate when the mean value became significantly different from day 3; ANOVA, and LSD, P < 0.05.
Figure 17. Salbutamol did not significantly improved tetanic $[Ca^{2+}]_i$ at resting fibers. MEM was supplemented with 10% FBS, 300 µU/ml insulin and 50 µM AraC. Salbutamol and DMSO concentrations were respectively 5 µM and 0.1% (v/v). 50% of culture medium was changed every 2 days. Fibers were plated on glass coverslip coated with collagen/laminin. Tetanic $[Ca^{2+}]_i$, defined as $[Ca^{2+}]_i$ during contraction was recorded when fibers were stimulated with 200 ms train of 10 V pulses at indicated frequency. Vertical bars represent the S.E. of 5 mice. There were no significant differences between the three experimental conditions, ANOVA $P>0.05$. 
Figure 18. Salbutamol did not improve tetanic \( [Ca^{2+}]_i \) during chronic stimulation. MEM was supplemented with 10% FBS and 300 µU/ml insulin. Salbutamol and DMSO concentrations were respectively 5 µM and 0.1% (v/v). 50% of culture medium was changed every 2 days. Fibers were plated on glass coverslip coated with collagen/laminin. Stimulation in culture was 50 ms long train of pulses at 80 Hz every 15 min. Tetanic \( [Ca^{2+}]_i \), defined as \( [Ca^{2+}]_i \) the during contraction was recorded when fibers were stimulated with 200 ms train of 10 V pulses at indicated frequency. Vertical bars represent the S.E. of 5 mice.

† Indicate when the mean value became significantly different from day 3.

* Mean value from control was significantly different from mean value of salbutamol and DMSO; ANOVA, and LSD, P < 0.05.
DISCUSSION

The major findings of this study were: 1) under resting condition MEM was a better culture medium than DMEM. 2) Inhibition of satellite cell and or fibroblasts mitosis with AraC improved fiber survival under resting condition but not when fibers were chronically stimulated. 3) Change in culture media solution every 2 days prolonged fiber survival compared to changing the medium every day. 4) For resting fibers, Matrigel was better than collagen/laminin in the absence of AraC, whereas the reverse was true in the presence of AraC. 5) Chronic stimulation worsened the loss of fibers. 6) Addition of insulin or taurine slightly improved fiber survival when chronically stimulated. 7) Salbutamol did not improve fiber survival. 8) Neither chronic stimulation nor salbutamol significantly affected tetanic [Ca$^{2+}$]. 9) Under resting conditions, the stimulation strength curve was shifted toward high voltage values whereas it shifted toward lower voltages when fibers were chronically stimulated.

OPTIMIZATION OF SINGLE FDB FIBERS IN CULTURE

Culture medium MEM vs DMEM

Despite the fact that DMEM contains more nutrients than MEM, fibers survived better in culture when MEM is used. This result coincides with the fact that FDB fibers have better morphology, contractile and fatigue characteristics when they are separated by trituration, following a collagenase digestion in MEM, rather than in DMEM (Selvin et al., 2014). As discussed by Selvin, the problem with DMEM is its osmolarity. MEM osmolarity is 319 mOsmoles whereas for DMEM it is 369 mOsmoles. Considering an intracellular osmolarity of 290 mOsmoles, then DMEM creates a greater osmotic shock than MEM, which, over days in culture, may well be reducing fiber survival.
**Satellite cell proliferation and fiber survival**

This study shows for the first time that AraC, a mitotic inhibitor, greatly enhanced fiber survival in culture under resting conditions especially in MEM changed every 2 days. It first slowed down the disappearance of fibers with normal morphology; that is, after 14 days in culture there were no fibers with normal morphology in the absence of AraC versus 62% in its presence at day 17. It also abolished the appearance of degenerating fibers, albeit it increased the number of supercontracted fibers.

Adult muscle fibers are non-mitotic cells, and AraC does not inhibit mRNA synthesis (Troy et al., 2012). It is therefore unlikely that AraC affects muscle fibers directly. However, there are small cells, known as satellite cells, which are skeletal muscle specific stem cells located on the surface of muscle fibers (Bischoff, 1975, 1986a; Rosenblatt et al., 1994; Danoviz & Yablonka-Reuveni, 2012). Satellite cells are believed to be mitotically quiescent in resting adult muscle, but they re-enter the cell cycle following muscle fiber damage to generate myoblasts, which subsequently fuse to form new fibers (Bischoff, 1975; Borisov et al., 2001; Schmalbruch, 2006). Several studies have reported that satellite cells are quickly activated when single muscle fibers are placed in culture, entering the cell cycle and expressing, within 24 hrs, a regenerating factor such as MyoD, which is myogenic factor (Yablonka-Reuveni & Rivera, 1994; Cornelison & Wold, 1997). Satellite cells of EDL and soleus rapidly migrate away from fibers while, for FDB fibers, they remained attached to the fiber for up to 2 days (Rosenblatt et al., 1996; Tavi et al., 2010; Keire et al., 2013).

In this study, mononucleated cells, which were possibly satellite cells or fibroblasts, were eventually observed when fibers were cultured for a few days in MEM in the absence of AraC (Fig.1D). These cells were not observed when AraC was added (data not shown). This suggests
that AraC most likely inhibits satellite cell and fibroblasts proliferation. This then raises two questions: i) why are these cells activated in fibers with normal morphology and contractility, i.e. with no evidence of fiber damage, and ii) are these cells once activated contributing to the degeneration of fibers in culture? In regard to the activation of satellite cells, one possibility is that the long preparation that includes dissection, collagenase digestion, trituration and plating, may stress fibers and still inadvertently activate satellite cells. In fact, it is hard to apply this protocol without stressing fibers. It may also be due to factors released by damaged or degenerating fibers. Fiber damage results in the release of growth factors, such as BDNF and HGF which are involved in the activation of quiescent satellite cells (Bischoff, 1986b; Tatsumi et al., 1998). Furthermore, adding crushed muscle extract allows satellite cells to divide after 16 h, an effect that is stopped when the extract is withdrawn (Bischoff, 1986a, 1986b). So, any factors released during fiber preparation or from degenerated or supercontracting fibers may be activating satellite cells.

Regardless of the activation mechanism, the inhibition of satellite cell (and fibroblast) proliferation by AraC suggests that these cells may contribute to the degenerating process of muscle fibers, especially because, in the presence of AraC, there was no degenerating fiber. In addition, one study reported that quiescent satellite cells when still attached to muscle fibers express anti-apoptotic factors (survival protein) such as Bcl-2 in culture (Yang et al., 1995; Jejurikar SS1, Marcelo CL, 2002; Adhihetty et al., 2007). Another study reported that Bcl-2 is expressed in adherent satellite cells to muscle fibers even after 10 weeks of denervation but not in non-adherent satellite cells in culture (Jejurikar SS1, Marcelo CL, 2002). So, one possible explanation for the lack of degenerating fibers in the presence of AraC is that satellite cells continue to express Bcl-2 to prevent apoptosis in muscle fibers.
Denervated muscle in situ are prone to contracture as cell membrane depolarizes and as small conductance Ca\(^{2+}\) sensitive K\(^{+}\) channels are expressed (Neelands et al., 2001). The same is then expected to occur in culture, except that in the absence of any attachment, uncontrolled and extreme contracture may lead to the extreme shortening observed with supercontracted fibers, a non-reversible state. The great number of contracting fibers in the presence of AraC, rather than in the absence of AraC, may be related to a lower number of degenerating fibers resulting in a great number of intact fibers that eventually supercontract.

Contrary to the situation with resting fibers, the addition of AraC did not improve fiber survival of chronically stimulated fibers. In fact, the addition of AraC worsened fiber survival, and the effect was statistically significant. In addition to the importance of satellite cells in regeneration following injury, they play a role in the adaptive response of skeletal muscle to exercise. Resistance training results in fiber hypertrophy (MacDougall et al., 1980). As fiber size increases, the number of myonuclei within a fiber must increase to maintain a constant nucleus domain (Rosenblatt et al., 1994). This occurs through satellite cell activation (Rosenblatt et al., 1994; Allen et al., 1999; Vierck et al., 2000; Hawke, 2005). A study has shown that there was increased in satellite cell activation and proliferation in adult muscle after resistance exercise (Vierck et al., 2000). Early work has demonstrated that proliferating satellite cells eventually fuse to existing fibers during hypertrophy to introduce nuclei into fiber. Overall these studies provide strong evidence for the activation of satellite cells with muscle activity due to some cross talk between fibers and satellite cells. If exercise activates satellite cells, then it is possible that the chronic stimulation also increases cross talk between fibers and satellite cells in culture. However, the survival of chronically stimulated fibers was barely 5 days and may have been too short to see any real effect from AraC. So, at this point it is not surprising that the survival of chronically
stimulated fibers is not improved by AraC. However, considering that chronically stimulated fibers only survived a few days, these experiments will have to be repeated once survival time is prolonged to make sure that abolishing satellite cell and/or fibroblast proliferation really worsened survival.

**Adhering substance and frequency of changing the culture medium solution: importance of myokines**

Change in medium is important to remove metabolic end products such as lactate. Interestingly, fiber survival was greatly improved when 50% of the culture media was changed every 2 days instead of every day. The most likely explanation for this effect is that frequent 50% medium changes constantly diluted myokines released by fibers or cytokines by other cells, which improves fiber survival. As discussed in the Introduction, nerve growth factor (NGF) is one myokine that improves muscle regenerating capacity in dystrophic muscle (Capsoni et al., 2000). IGF-1 and BDNF, two other myokines, induce hypertrophy either by promoting protein synthesis or by activating satellite cells (Vandenburgh et al., 1991; Clow & Jasmin, 2010). So, in future studies it will be important to document the nature of all myokines and cytokines that are released in culture and then determine which ones are the most important for fiber survival. It will also be interesting to determine how the amounts of myokines and cytokines release in culture differ from those in situ. For example, addition of NGF, IGF-1 and BDNF to culture medium either individual or in combination, may prolong fiber survival due to their anabolic effect.

The importance of exogenous factors was also observed in experiments in which fibers were plated on just collagen/laminin or on Matrigel, as the latter contained several growth factors. In the absent of AraC, fiber survival was much better with Matrigel than collagen/laminin (Fig.5). The presence of growth factors in Matrigel probably either counteracted the degeneration induced
by satellite cells such as IGF or replaced the lack of anti-apoptotic factors such as Bcl-2 (Jejurikar SS1, Marcelo CL, 2002). It was therefore surprising that in the presence of AraC the full absence of any factors did not improve fiber survival; in fact fiber survival was better with just collagen/laminin. Perhaps, in the absence of fiber degeneration a greater number of normal secreted their own myokines, which may have also been more suitable for fiber survival. Regardless of the mechanisms, the better fiber survival with collagen/laminin as the adhering substrates in the presence of AraC, is an advantage to better control which factors fibers are exposed to.

**CHRONIC STIMULATION DOES NOT IMPROVE FIBER SURVIVAL IN CULTURE**

Several in vivo studies have shown that chronic stimulation reverses atrophy of denervated skeletal muscle (MacDougall *et al.*, 1980; McCall *et al.*, 1996; Ashley *et al.*, 2007a). However, in culture chronic stimulation had the reverse effect. Regardless of the stimulation protocols and under the best culture conditions, chronically stimulated fiber survival was significantly diminished to 8 days (Fig. 9) compared to 21 days for resting fibers (Fig. 5). Degenerating fibers were not observed with chronic stimulation, but the number of supercontracted fibers reached 100% by day 11 whereas at rest supercontracting fibers did not exceed 33% for up to 17 days.

As mentioned in the Introduction, denervation induces a reduction in resting membrane potential ($E_M$); i.e., a depolarization (Albuquerque *et al.*, 1971; Vyskočil *et al.*, 1973; Bray *et al.*, 1976). For resting fibers, there was a shift in the a number of contracting fibers-stimulus strength to higher voltage. This shift is most likely because the depolarization increased the number of Na$^+$ channels that became inactivated (Goldman, 1995). Contrary to resting fibers, chronic stimulation shifted the number of contracting fibers-stimulus strength relationship toward lower voltages, which is expected to reduce the difference in resting membrane potential and the threshold.
potential at which action potential is triggered. As mentioned above, denervated muscles are prone to contracture. So, the reduced difference in resting $E_M$ and threshold potential in chronically stimulated fibers resulted in more action potentials being triggered compared to the situation in resting fibers for which the difference in potential increased (Bray et al., 1976). Finally, although the Ca$^{+2}$ release is significantly lower during tetanic contraction within one day in culture, any prolonged contraction may eventually allow $[\text{Ca}^{+2}]_i$ to increase to a point at which a large and irreversible supercontraction occurs.

Insulin did not improve fiber survival or lower the number of supercontracted fibers, suggesting that an energy deficiency is not responsible for the supercontraction in chronically stimulated fibers. While insulin activates the Na$^+$/K$^+$-ATPase pump and taurine helps in stabilizing resting membrane potential, none of them lowered the number of supercontracted fibers. In fact, insulin and taurine improve the number of contracting fibers-stimulus strength relationship at day 3 compared to control conditions, but such improvement was too small to consider any physiological significance in fiber survival.

THE EFFECT OF SALBUTAMOL ON FIBERS

Salbutamol is a $\beta_2$ agonist that mimics the actions of naturally occurring catecholamines. $\beta_2$-adrenoceptor agonists, such as salbutamol and clenbuterol have the ability to increase skeletal muscle mass in denervated muscle (Zeman et al., 1987; Lynch & Ryall, 2008). They have actually been considered as a potential therapeutic approach for treatment of muscle atrophy (Lynch & Ryall, 2008).

It was therefore expected that salbutamol would improve fiber survival in culture; this was however not the case. In resting conditions, fibers with normal morphology had disappeared by 17 days in the presence of salbutamol, compared to 19% of fibers with normal morphology by 21 days.
in control condition. For chronically stimulated fibers, both DMSO, the vehicle, and salbutamol lowered fiber survival. The reason as to why salbutamol fails to improve fiber survival in culture cannot be explained from the results of this study or from other studies, except to suggest that the intracellular signaling pathways downstream of the β2-receptors are modified differently in culture vs. denervated fibers in situ. With regard to the DMSO effect with chronic stimulation, there is evidence that prolonged exposure to DMSO produces damage in T-tubules (Fraser, 2011), and that this damage may be exaggerated by chronic stimulation.

Several studies illustrated the effect of short stimulation of β2 adrenergic receptors on force production in isolated skeletal muscle (Siedlecka et al., 2008; McCormick et al., 2010) whereas long stimulation of β2 adrenergic receptors has never been investigated in vitro. Nevertheless, acute exposure to β2 adrenergic agonist (salbutamol) increases force production in slow and fast twitch muscle in situ (McCormick et al., 2010). For resting fibers in culture, salbutamol did not affect tetanic [Ca^{2+}]i upon stimulation to contract (Fig.17), as the reductions in the tetanic [Ca^{2+}]i at various stimulation frequencies at day 7 and day 14 for fibers exposed to salbutamol, were not different than the reductions from control fibers. So, while acute exposure to β2 agonists increases the force of innervated muscles in situ, a chronic exposure of denervated fibers in culture has no effect on tetanic [Ca^{2+}]i and thus no effect on force.

In conclusion, this study provides a new protocol to study adult muscle fibers in culture. For resting fibers, this study demonstrated that blocking the proliferation of satellite cells (and fibroblasts) with AraC is crucial for the fibers to survive longer periods of time, mainly because it appears to prevent any degeneration process. However, AraC did not decrease fiber loss due to supercontractions. It will be interesting to determine in future studies whether partially reducing Ca^{2+} release with dantrolene, an inhibitor of the Ca^{2+} release channel of sarcoplasmic reticulum,
can prevent supercontraction and further improve fiber survival. It was also found that, in the presence of AraC, the coverslip only needs to be plated with collagen/laminin, which provided a better survival condition than Matrigel. It was proposed that fibers release myokines that are better suited for fiber survival than the growth factors present in Matrigel. This notion was further supported by the fact that changing 50% of the culture medium every 2 days largely improved fiber survival compared to changing the medium every day, because the latter most likely removed the myokines. Contrary to the in situ observation, neither chronic stimulation nor salbutamol, a β2 adrenergic receptor, improved fiber survival and contractility of fibers in culture. Finally, it is proposed that the next step is to analyze which myokines are released by fibers in culture and to compare the results with those from innervated and denervated fibers in situ. Once the nature of the myokines are known, then they can be investigated to determine which ones alone or in combination improves fiber survival and contractility in muscles, as well as to determine if some are involved in muscle plasticity.
Appendix I. No significant difference in fiber survival between plated fibers on glass coverslip or plastic dish plated with just collagen instead of Matrigel. Fibers were cultured in MEM supplemented with 10% FBS. 50 % of culture medium solution was changed every day. A) Number of fibers with normal morphology. B) Number of degenerating fibers. C) Number of supercontracted fibers. Numbers of fibers are expressed as a percent of total number of fibers present on coverslip the day they were counted. D) Mean total number of counted fibers per mice. Vertical bars represent the S.E. of 4 mice.

§ Indicate when the mean value became significantly different from 100% in A or 0% in B and C; ANOVA, and LSD, P < 0.05.
Appendix II. AraC was improved fiber survival with collagen when plastic dish were used instead of glass coverslips. Fibers were kept in MEM supplemented with 10% FBS without or with 50 µM AraC. Fibers were plated on plastic dish coated with either collagen/laminin. 50% of culture media were changed every 2 days. A) Number of fibers with normal morphology. B) Number of degenerating fibers and C) supercontracted fibers. D) Mean total number of counted fibers per mice. Vertical bars represent the S.E. of 2-6 mice.

§ Indicate when the mean value became significantly different from 100% in A or 0% in B and C.
† Mean value from using AraC was significantly different from mean value no AraC; ANOVA, and LSD, P < 0.05.
Appendix III

Collagen/Laminin slightly improved chronic stimulated fiber survival than Matrigel. Fibers were cultured in MEM supplemented with 10% FBS. Glass coverslip and plastic culture dish were covered with collagen/laminin (A-C) or Matrigel (D-F). 50% of culture medium solution was changed every day. Fibers were chronically stimulated with 100 ms train of pulses at 20 Hz every 15 min. A, D) Number of fibers with normal morphology. B, E) Number of supercontracted fibers. Numbers of fibers are expressed as a percent of total number of fibers present on coverslip the day they were counted. C, F) Mean total number of counted fibers per mice. Vertical bars represent the S.E. of 2-4 mice.

§ Indicate when the mean value became significantly different from 100% in A and D or 0% in B and D; ANOVA, and LSD, P < 0.05.
# Appendix IV

Table A.1: Minimum essential medium (MEM) formulation (EAGLE et al., 1959).

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Table A.2: Dulbecco’s modified eagle medium (DMEM) formulation (DULBECCO & FREEMAN, 1959).

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<td>210</td>
<td>42</td>
<td>0.2</td>
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<tr>
<td>L-Isoleucine</td>
<td>131</td>
<td>105</td>
<td>0.802</td>
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<tr>
<td>L-Leucine</td>
<td>131</td>
<td>105</td>
<td>0.802</td>
</tr>
<tr>
<td>L-Lysine hydrochloride</td>
<td>183</td>
<td>146</td>
<td>0.798</td>
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<tr>
<td>L-Methionine</td>
<td>149</td>
<td>30</td>
<td>0.201</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>165</td>
<td>66</td>
<td>0.4</td>
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<tr>
<td>L-Serine</td>
<td>105</td>
<td>42</td>
<td>0.4</td>
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<tr>
<td>L-Threonine</td>
<td>119</td>
<td>95</td>
<td>0.798</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>204</td>
<td>16</td>
<td>0.0784</td>
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<tr>
<td>L-Tyrosine disodium salt dehydrate</td>
<td>261</td>
<td>104</td>
<td>0.398</td>
</tr>
<tr>
<td>L-Valine</td>
<td>117</td>
<td>94</td>
<td>0.803</td>
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<tr>
<td><strong>Vitamins</strong></td>
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<td></td>
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<tr>
<td>Choline chloride</td>
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<td>4</td>
<td>0.0286</td>
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<tr>
<td>D-Calcium pantothenate</td>
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<td>4</td>
<td>0.00839</td>
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<tr>
<td>Folic Acid</td>
<td>441</td>
<td>4</td>
<td>0.00907</td>
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<tr>
<td>Niacinamide</td>
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<td>0.0328</td>
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<tr>
<td>Pyridoxine hydrochloride</td>
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<td>Riboflavin</td>
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<td>0.00106</td>
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<tr>
<td>Thiamine hydrochloride</td>
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<tr>
<td>i-Inositol</td>
<td>180</td>
<td>7.2</td>
<td>0.04</td>
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<td><strong>Inorganic Salts</strong></td>
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<tr>
<td>Calcium Chloride (CaCl2) (anhyd.)</td>
<td>111</td>
<td>200</td>
<td>1.8</td>
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<tr>
<td>Ferric Nitrate (Fe(NO3)3*9H2O)</td>
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<td>0.1</td>
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<tr>
<td>Magnesium Sulfate (MgSO4) (anhyd.)</td>
<td>120</td>
<td>97.67</td>
<td>0.814</td>
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<tr>
<td>Potassium Chloride (KCl)</td>
<td>75</td>
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<td>Sodium Bicarbonate (NaHCO3)</td>
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<td>3700</td>
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<td>Sodium Chloride (NaCl)</td>
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<td>6400</td>
<td>110.34</td>
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<td>Sodium Phosphate monobasic (NaH2PO4-H2O)</td>
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<td>125</td>
<td>0.906</td>
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<td><strong>Other Components</strong></td>
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<tr>
<td>D-Glucose (Dextrose)</td>
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<td>4500</td>
<td>25</td>
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<tr>
<td>Phenol Red</td>
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<td>Sodium Pyruvate</td>
<td>110</td>
<td>110</td>
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REFERENCES


Fraser JA (2011). Dimethyl sulphoxide addition or withdrawal causes biphasic volume changes and its withdrawal causes t-system vacuolation in skeletal muscle. J Physiol 589, 5555–6; author reply 5557.


Jejurikar SS1, Marcelo CL KWJ (2002). Skeletal muscle denervation increases satellite cell susceptibility to apoptosis.


Selvin D. Regulation of myoplasmic Ca2+ during fatigue in KATP channel deficient FDB muscle fibers (Dissertation). University of Ottawa, Ottawa, Ontario, Canada, 2013.


